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Interspecies-Transmission of Animal Coronaviruses

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For Tina, my love.

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List of abbreviations

Aa	Amino acid
ACE2	Angiotensin Converting Enzyme 2
APN	Aminopeptidase N
bp	Basepairs
BCA	Bicinchoninic acid
cDNA	Complementary DNA
CoV	Coronavirus
CO_2	Carbon dioxid
C-terminal	COOH terminus
Cy3	Indocarbocyanine
dNTP	Desoxynucleotide
DAPI	4´,6´-Diamidino-2-phenylindol
DEPC	Diethylpyrocarbonat
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
DPP4	Dipeptidylpeptidase 4
DTT	Dithiothreitol
et al.	Et alli
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle´s Modified Essential Medium
ER	Endoplasmatic Reticulum
FCS	Fetal Calf Serum
FCoV	*Feline Coronavirus*
g	Gramm or Gravitational force
GFP	Green Fluorescent Protein
HCoV	Human Coronavirus
HRP	Horse raddish peroxidase
IBV	Infectious Bronchitis Virus
IF	Immunofluorescence
IgG	Immunglobulin G
kb	Kilobases
kDa	Kilodalton
l	Liter
LB	Luria Bertani
mA	Milliampere
mg	Milligramm
ml	Milliliter
mRNA	Mesenger RNA
M	Molarity; molar
MHV	Murine Hepatitis Virus
MOI	Multiplicity of Infection
MW	Molecular weight
N-terminal	NH_2 terminus
pH	Potentia Hydrogenii
PBS	Phosphate buffered saline

PBSM	PBS without calcium and magnesium
PCR	Polymerase chain reaction
rpm	Rounds per minute
RNA	Ribonucleic acid
RT	Roomtemperature
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
taq	Therus aquaticus
TAE	Tris-Acetate-EDTA
TBE	Tris-Borat-EDTA
Tris	Tris(hydroxymethyl)aminoethan
U	Unit [µmol/min]
V	Volt
VSV	*Vesicular Stomatitis Virus*

Interspecies-Transmission of Animal Coronaviruses

Tim Gützkow

1 Summary

In recent years many emerging viruses threatening human health were discovered and found to have their major host reservoir in bats. Rabies, Ebola, Henipah and Coronaviruses are the most prominent under these zoonotic pathogens, where especially the emergence of severe acute respiratory syndrome (SARS) coronavirus in 2002 and the recent appearance of Middle East respiratory syndrome (MERS) coronavirus had gained global awareness. Great effort has been invested to uncover the course of events of their introduction to the human population. Coronaviruses may be exemplary for many zoonotic RNA viruses, so that the study of their genesis expected to provide insights into basic questions about viral zoonosis. For coronaviruses the recognition of a specific receptor by the viral glycoprotein appears to be a major constrain of interspecies transmission. Therefore, it is important to address the question whether a large shift in receptor specificity was necessary for their transmission to humans. The closest related relative to SARS coronavirus was identified in bats of the genus *Rhinolophus* in South-East of China, but until today no coronavirus was isolated from bats. What is known is that these viruses are not able to utilise the same receptor as the human SARS coronavirus, the human angiotensin converting enzyme *2* (ACE2).

The aim of our studies was to identify the receptor of these bat SARS-like coronaviruses, which would help to estimate the likelihood of their transmission to humans. We therefore used three different glycoproteins of bat SARS-like coronaviruses isolated from *Rhinolophus* bats in China, Bulgaria and Spain and tried to identify their cellular receptors analysing cell lines of 14 different bat species, in binding as well as infection assays. Unfortunately neither binding nor infection could be observed for the spike proteins tested. We also tested known coronavirus receptors like human ACE2, aminopeptidase N (APN) and dipeptidylpeptidase 4 (DPP4) and successfully cloned *Rhinolophus* ACE2 and DPP4. None of these proteins facilitated binding or infection in transient expression. This indicates that bat SARS-like coronaviruses utilise a novel coronavirus receptor.

In contrast, we could show that SARS coronavirus can utilise ACE2 of two *Rhinolophus* species living in Europe, indicating that a proposed switch in receptor specificity may not be obligatory for the precursor of SARS-CoV to cross the species barrier. It may

further suggests that bats are reservoir to at least two different lineages of coronaviruses, which differ in their receptor usage.

Interspezies-Transmission von tierischen Coronaviren

Tim Gützkow

2 Zusammenfassung

In den letzten Jahren wurde eine Vielzahl an unbekannten Viren in Fledermäusen entdeckt die eine Bedrohung für die menschliche Gesundheit darstellen. Tollwut, Ebola, Henipah und Coronaviren sind die bekanntesten darunter, wobei gerade das SARS Coronavirus in 2002 sowie das kürzlich aufgetauchte MERS Coronavirus weltweite Aufmerksamkeit erregten. Große Bemühungen wurden angestrengt um aufzuklären auf welchem Weg sie in die menschliche Population gelangen konnten. Coronaviren könnte hierbei als Vorbild für viele verschiedene RNA-Viren dienen, so dass die Analyse ihrer Entstehung Einblicke liefern könnte in grundsätzliche Fragen über zoonotische Viren. Für Coronaviren scheint die Erkennung eines speziellen Rezeptors durch das virale Glykoprotein eine entscheidene Barriere für die interspezies Übertragung zu sein. Deshalb ist es wichtig zu fragen ob eine Verschiebung der Rezeptor Spezifität notwendig war um auf Menschen übertragen zu werden. Der nächste Verwandte des SARS Coronavirus wurde identifiziert in Fledermäusen der Gattung *Rhinolophus* im Süd-Osten Chinas, aber bis heute konnte noch keine Virus aus Fledermäusen isoliert werden. Es ist aber bekannt das diese Viren nicht in der Lage sind denselben Rezeptor wie das humane SARS Coronavirus zu verwenden, dass humane *Angiotensin Converting Enzyme 2* (ACE2).

Ziel unserer Studien war die Identifikation des Rezeptors dieser SARS-ähnlichen Coronaviren der Fledermäuse, was uns dabei helfen könnte die Wahrscheinlichkeit einer Übertragung einzuschätzen. Wir nutzten dafür drei verschiedene virale Glykoproteine solcher SARS-ähnlicher Fledermaus Coronaviren, welche aus Rhinolophus Fledermäusen in China, Bulgarien und Spanien identifiziert wurden. Mit diesen Proteinen habe wir versucht zelluläre Rezeptoren in 14 verschiedenen Fledermaus Arten zu identifizieren, und dabei sowohl Bindungs- als auch Infektionsexperimente angewendet. Unglücklicherweise konnten wir weder Bindung noch Infektion nachweisen. Wir testeten zusätzlich bekannte Coronavirus Rezeptoren wie ACE2, APN und DPP4, sowie darüber hinaus erfolgreich isolierte *Rhinolophus* ACE2´s und DPP4. Keines dieser Proteine führte zu Bindung oder Infektion wenn sie transient expremiert wurden. Dies deutet an das SARS-ähnliche Coronaviren in Fledermäusen einen bisher unbekannten Coronavirus Rezeptor verwenden.

Überraschenderweise belegten unsere Ergebnisse das das SARS Coronavirus in der Lage ist die ACE2 Proteine zweier europäischer Rhinolophus Arten zu nutzen, was

andeutet das der angenommene Wechsel in der Rezeptor Spezifität nicht unbedingt nötig war für den Vorläufer des SARS Coronavirus, um die von einer Spezies auf die andere übertragen zu werden. Darüber könnte es bedeuten das Fledermäuse mindesten zwei verschiedene Arten von SARS-ähnlichen Coronaviren beherbergen, wovon eine ACE2 verwendet und die andere nicht.

3 Introduction

3.1 Coronaviruses

3.1.1 Taxonomy

The *Virus of Infectious Bronchitis* (IBV) reported by Hudson and Beaudette in 1932[73, 87] was the first description of a Coronavirus (CoV). After the additional discovery of the *Murine Hepatitis Virus* (MHV) and the *human Coronavirus 229E* (HCoV-229E) a group of virologist around J.D. Almeida and D.A.J. Tyrrell proposed these viruses as members of a new taxonomic group in 1968[1, 88]. In 1975, the family *Coronaviridae* became officially recognized by the *International Committee on Taxonomy of Viruses*[104, 160]. As viruses with a single stranded RNA genome of positive polarity they belong to the order *Nidovirales,* together with the families of *Arteriviridae*, *Mesoniviridae* and *Roniviridae*[20, 45, 114, 115]. The family *Coronaviridae* comprise the subfamilies of *Coronavirinae* and *Torovirinae*[19, 118, 124]. After a recent re-evaluation the *Coronavirinae* were separated into the genera *Alpha*-, *Beta*-, *Gamma*- and *Deltacoronavirus*. Coronaviruses infect a broad range of avian and mammalian hosts. Alpha- and betacoronaviruses are found exclusively in mammals where gamma- and deltacoronaviruses are predominantly found in birds and only to a minor extent in mammals (table 1).

Table 1: Coronavirus hosts

Subfamily	Hosts
Alphacoronavirus	bats, cats, humans, rabbits, pigs
Betacoronavirus	bats, cattle, dogs, horses, humans, mice, rats, pigs
Gammacoronavirus	birds, whales
Deltacoronavirus	birds

The last decade has been very productive in coronavirus research in respect to the discovery of new coronaviruses and the phylogenetic analysis of known genomes. Today there is a comprehensive model about the relationship and ancestry of these viruses, as shown in figure 1. According to this model, alpha- and betacoronaviruses share a common ancestor which most likely infected bats and the broad range of viruses infecting mammalian species of such diversity arose from interspecies transmission. Gamma- and deltacoronaviruses are assumed to have a common

ancestor who most likely infected birds and were later introduced into some mammals. There is no strong evidence indicating whether the ancestor of all four lineages infected birds or mammals.

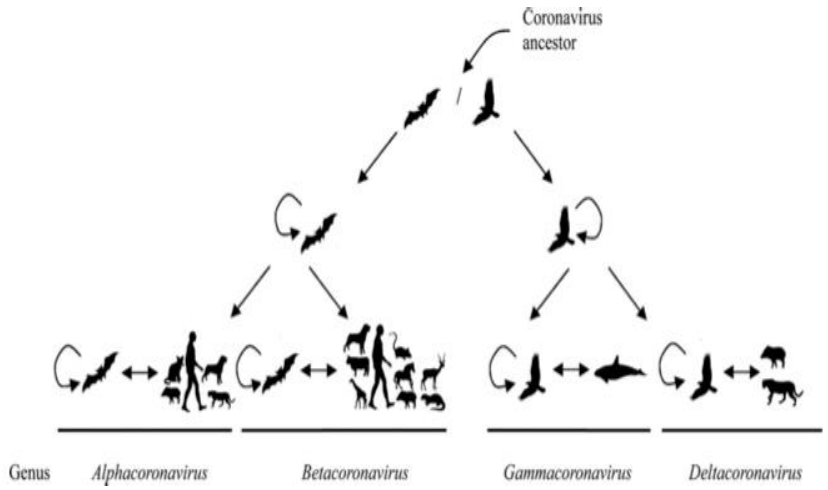

Figure 1: Coronavirus ancestry
Woo et al. 2012[173]

3.1.2 Morphology

Large protrusions from the viral surface are the characteristic features of these viruses when analysed by electron microscopy and resulted in the designation “Coronavirus”. The particles are enveloped and of pleomorphic, mostly spheroid appearance with a diameter of 80-160 nm. The viral genome is tightly encapsidated in a shell made up from the nucleocapsid protein (N). This complex of nucleic acid and protein is designated ribonucleoprotein (RNP). The N protein is indispensable for viral assembly and its three-dimensional structure is so essential that its amino acid composition is one of the most highly conserved ones under all viral proteins. Bound to the RNA by a specific interaction site, it mediates the connection of the core and the viral envelope by interacting with the membrane protein (M). The M protein is inserted in the viral envelope and binds the

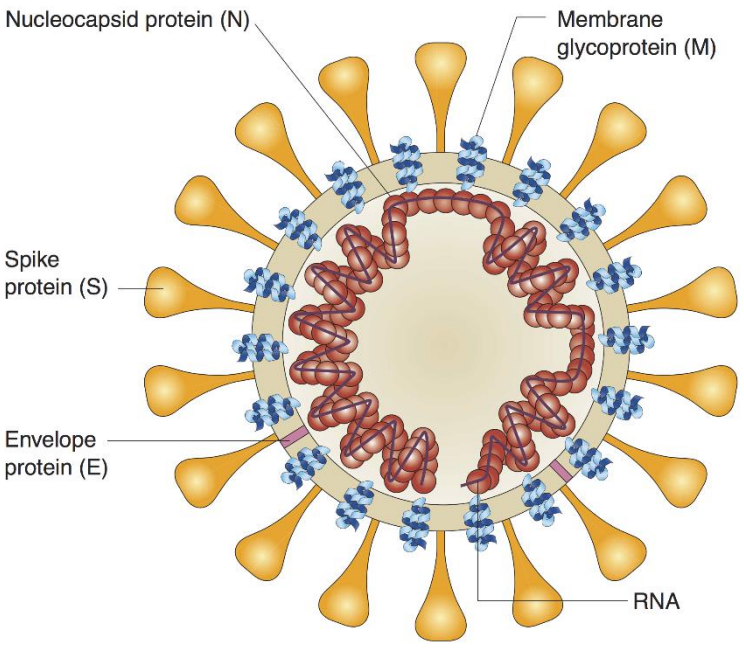

Figure 2: Coronavirus structure
Structure of a coronavirus particle without the HE-protein. Peiris *et al.* 2004[112]

N proteins as well as the spike protein (S)[109] and is therefore as essential as the N protein for the overall structure of the viral particle by determining the position of its components. Beside the M and S proteins, two other viral proteins can be found in the viral envelope, the envelope protein (E) and for some betacoronaviruses an additional hemagglutinin-esterase protein (HE). From all structural proteins, the E protein appears to be the only structural protein which is not completely indispensable[82]. Its function is not fully elucidated but it appears to be involved in the assembly as it has been shown the combined expression of M and E protein results in the formation of virus like particles.

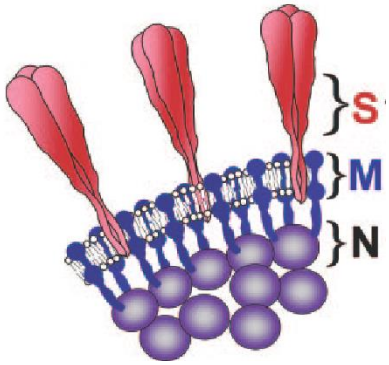

Figure 3: Proposed interaction of the S, M and N proteins
Neuman *et al.* 2006[108]

The HE protein is a peculiarity just found in some but not all betacoronaviruses. In *Ortho-* and *Paramyxoviruses* a known analogue features a neuraminidase activity which cleaves sialic acids residues from surface sialoglycoconjugates and in this way helps progeny virus particles to be released from the host cell. For the coronavirus HE protein, a related enzyme function is assumed to facilitate the early stages of viral entry[135, 161].

3.1.3 Coronavirus spike protein

The ability of coronaviruses to infect a specific host cell is determined by the spike protein[6, 18, 31, 32, 62, 81, 131, 149, 157, 159]. During a coronavirus infection humoral reactions are mainly directed against this protein[27, 145, 170 15]. It is a class I transmembrane protein which forms homotrimers in the viral envelope[33]. Biochemical and cryo-electron microscopy studies indicate a number of about 70-100 spike trimers on the surface of an average coronavirus particle[61]. Variable in length from 1,160 amino acids for IBV up to 1,400 amino acids for the *Feline Coronavirus* (FCoV), this protein is highly glycosylated with 21 to 35 potential *N*-glycosylation sites and has a molecular weight of about 180-200 kDa. Two functional domains can be distinguished on the large ectodomain. One (S1), at the amino-terminal end, mediates attachment and binding to a receptor whereas the second one (S2) is responsible for the fusion of the viral and the host cell membrane. The S2 domain is the most conserved part of this protein and contains a fusion peptide as well as two regions of heptad repeats. These are repeated

heptapeptides with every first and fourth amino acid being a hydrophobic and every fifth and seventh being a charged residue. These repeats form α–helices and are characteristic for coiled-coil secondary protein structures. The fusion peptide is located close to the N-terminal end of this domain. This peptide is inserted into the target membrane after a conformational change of the spike protein and serves as an anchor. Spike proteins share all these features with other class I fusion proteins, e.g. those of members of the families *Retroviridae*, *Filoviridae* or *Paramyxoviridae*, but coronaviruses differ in one important aspect. Whereas most other class I fusion proteins are cleaved by a cellular protease site which is cleaved during maturation into two subunits, this is only reported for two coronavirus genera, the gamma- and deltacoronaviruses. Most alpha- and betacoronaviruses appear to contain uncleaved spike proteins incorporated in their matured virus particles[51, 70]. They may be cleaved once they reach endosomes in the host[7, 10, 11, 13, 53, 72, 101, 117, 140, 141]. Another difference is that the position of the coronavirus fusion peptide is about 200 residues away from the proposed cleavage site[13].

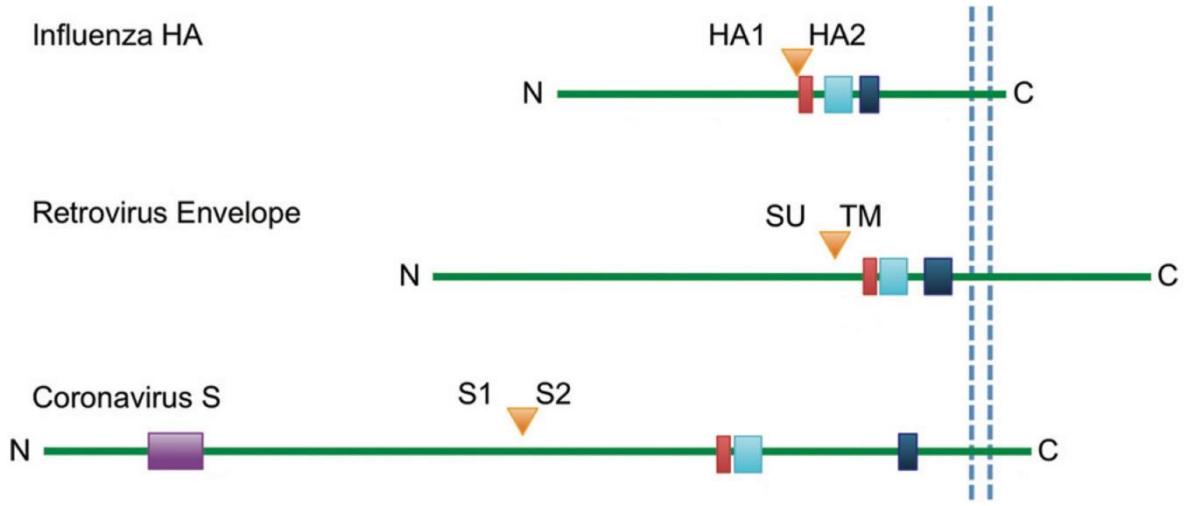

Figure 4: Class I fusion proteins
Comparison of three class I viral fusion proteins. Orange triangle = proteolytic cleveage site; red bar = fusion peptide; light blue bar = heptad repeat 1; dark blue bar = heptad repeat 2; violette bar = RBD of MHV. *Graham et al. 2010*[59]

The S1 domain surrounds the stalk-like structure of the joined S2 domains within the homotrimer. It is the portion which interacts with the host cell directly and thus mediates attachment and binding. In contrast to the S2 domain, this S1 varies considerably between different coronaviruses and even between different strains of the same species, as observed for the *Murine Hepatitis Virus* (MHV). Some coronaviruses utilize sialic acids of cell surface components as binding partners[133, 134, 136, 162, 171], others recognize a specific protein receptor[34, 44, 67, 92, 121, 156, 179], in either case binding is

mediated by the S1 domain. For some coronaviruses it was even possible to identify discrete domains of 180-330 amino acids, which are independently folded and specifically interact with the respective receptor, designated Receptor Binding Domains (RBD)[4, 12, 14, 80, 172, 177 165]. Although the location of the RBDs differs considerably between coronavirus species. While the RBD of MHV and the porcine *Transmissible Gastroenteritis Virus* (TGEV) are located at the N-terminus of the S1 domain[77, 80, 113], for all other coronaviruses the binding site is close to S1/S2 cleavage site[14, 35, 55, 68, 91, 96, 172, 175 165]. By crystallisation of RBDs bound to the specific receptor even the identification of individual binding partners at amino acid level has been solved for some coronaviruses[91, 113, 175, 178 165].

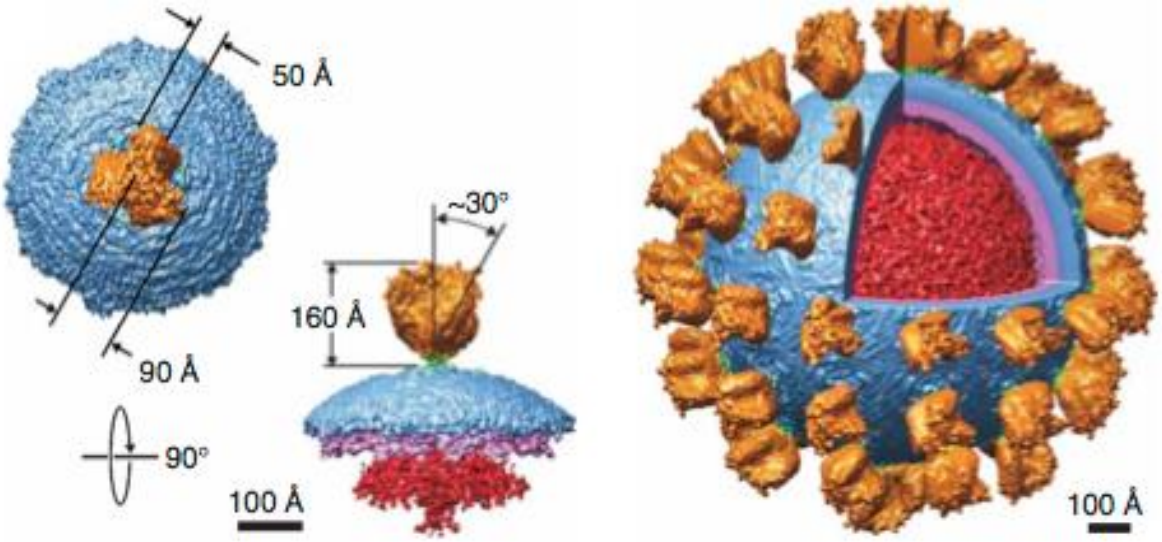

Figure 5: Cryo EM model of coronavirus particle
Red = nucleocapsid shell; violette = M protein; blue = lipid bilayer; green = spike S2 domain; orange = spike S1 domain.
Beniac et al. 2006[8]

Several coronavirus protein receptors have been identified, aminopeptidase N (APN)[34, 156, 179], carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1)[169], angiotensin converting enzyme 2 (ACE2)[67, 92] and dipeptidyltransferase 4 (DPP4)[121]. APN is a glycoprotein with metalloprotease activity, also known as CD13. It has a size of 150 kDa and is located at the plasma membrane. In addition to cell types of the lymphatic system and central nervous system, it can also be found in cells of the intestine and respiratory tract[75, 97, 138]. *Human coronavirus* 229E (HCoV-229E) utilizes human APN, TGEV porcine APN and FCoV feline APN. Interestingly, FCoV can only use fAPN as a receptor, whereas TGEV and HCoV-229E recognize no only pAPN or hAPN respectively, as a functional receptor, but in addition also the feline APN. This is

remarkable if one takes into account that the amino acid identity between feline APN and the human and porcine homologs is only about 77-78 % respectively[156].

CEACAM1 is expressed in a broad range of cell types including epithelial and endothelial cells. It is a glycoprotein localized at the plasma membrane and acts as a cell adhesion protein. This receptor can be found in liver and intestinal tissue which are also the main sites of MHV infection. But MHV is also able to use different smaller splicing products that are expressed at different organs, for example the brain[21, 181].

Two human coronaviruses employ ACE2 as a receptor, *Human Coronavirus NL63* (HCoV-NL63) and the *SARS Coronavirus* (SARS-CoV). The former one is a member of the genus *Alphacoronavirus* and the latter one belongs to the betacoronaviruses. Despite being only distantly related both viruses recognize the same epitope on the ACE2 molecules[95, 176]. ACE2 is type I transmembrane glycoprotein localized at the plasma membrane and belongs to the renin-angiotensin system (RAS), that plays a part in the regulation of blood pressure as well as balance of fluids and salts[79]. It is mainly expressed in the heart, kidneys and testes but also in lower levels lung, liver and intestine[64, 76, 151].

The *Middle East Respiratory Syndrome Coronavirus* (MERS-CoV) has been identified in 2012 in patients suffering from a severe respiratory infection. It utilizes DPP4[121], a protein expressed in almost all organs, as well as endothelial and epithelial cells. It is known to play a role in cell adhesion, nutrition and metabolism as well as the immune and endocrine system[58].

3.1.4 Genome

All nidoviruses have a complex genome structure, featuring nested transcription and ribosomal frameshifts. Their genome mimics the host messenger mRNA by having a CAP-structure as well as a 3´end polyadenylation, which allows them to be directly translated from the host cell ribosomes. At the 5´end of the coronavirus genome two Open Reading Frames (ORFs) are encoded. The first one is designated 1a and can be elongated by a programmed ribosomal frameshift of -1 to an ORF of the exceptional size of up to 20kb. Encoded in these two ORFs 1a/1ab are polyproteins which undergo autoproteolytic cleavage during and after their translation. The resulting proteins build up the viral RNA replication complex[57, 125, 127].

This RNA replication complex begins to synthesize full length copies of the genome that later on act as templates for the generation of genomes for the progeny virus particles and subgenomic RNAs for the translation of the structural proteins. The ORFs of coronaviruses are headed by an AU-rich motif of 10 nucleotide designated Transcriptional Regulatory Sequences (TRS)[5, 56, 84].

A model proposed by Sawicki *et al.* assumes that the transcription of the template happens in a discontinuous manner, where copies of variable length are produced. The probability that the transcription stops increases with the length of the transcript, whereas TRS also have some influence. The first appearing TRS on the 5´end at the newly transcribed subgenomic RNA now determines what ORF gets expressed, by modification of the leading nucleotide sequence through the replication complex[129, 130]. This model also correlates the quantities of viral structural proteins with the position of the respective ORFs in the viral genome, so that ORFs closer to the 3´end get more expressed. All known coronaviruses have the same sequence of ORFs encoding structural protein: 5´-replicase-(HE)-S-E-M-N-3´. Although this order appears highly conserved, experiments proofed that it is not vital as changes to it *in vitro* only led to impaired virus replication[30].

The size of coronavirus genomes of up to 32kb is exceptional when compared to all known RNA viruses and exceeds their genome size at least 10 times. This fact is astonishing when one takes into account that theoretical models propose an upper boundary for RNA genomes, which all other viruses conform to. This models are based on the lacking proofreading ability of RNA polymerases. It should lead to such a high mutation frequency that replication of RNA genomes of a certain length should no longer result in viable copies[39, 105]. Whereas it is not entirely unravelled, the *Coronaviridae*-specific ExoN protein may allow them to extend the limits by adding some sort of proofreading ability to the replication machinery. This was studied by a knock-out of this protein combined with an artificial increase of the mutagenic load. Thereby, the elimination of ExoN resulted in an increased mutation frequency during replication but the intact coronaviruses could withstand a mutation rate which was 18 times higher compared to others[38, 152].

They are many pieces of evidence that coronaviruses are prone to homologous recombination, a process where two nuclei acid molecules exchange material driven by a homology of the sequences at the point of interaction. As the TRS of even distantly related coronaviruses are very similar, they are destined as points of recombination

but it can also happen at other points of sequence homology. The analysis of some coronavirus genomes revealed so abundant recombination events that it is suspected that homologous recombination is a major force in coronavirus evolution[29, 89]. At a closer look coronavirus genomes show such a mosaic structure of shuffled elements, that interspecies exchange of genetic material seems to be the rule, rather than the exception[45, 78, 96, 124].

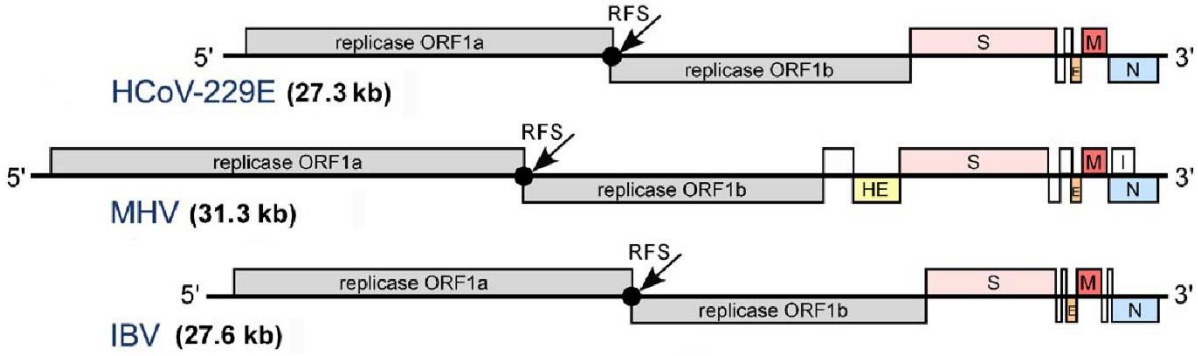

Figure 6: Comparison of coronavirus genome structures
Compared are the genomes of *human Coronavirus 229E*, *Murine Hepatitis Virus* and *Infectious Bronchitis Virus* (IBV). *Gorbalenya et al.* 2006 [57]

3.1.5 Replication cycle

The replication cycle of coronaviruses starts with the attachment of the viral particle to the cell surface, which is mediated by the spike protein. Studies indicate that the initial attachment involves cell-surface heparan sulphate proteoglycans and the actual binding to the protein receptor is a subsequent step[86]. Yet binding of the receptor is necessary for the following fusion of viral and cellular membrane, which is accomplished by an extensive conformational change of the spike protein.

Despite some studies reporting that viruses fuse at the cell surface[107], for most coronaviruses an uptake and transport to endosomes seems required to trigger this process, which may also involve cathepsins in case of alpha- or betacoronaviruses[72, 117, 140]. After fusion of the viral and the cellular membrane the viral nucleocapsid is released into the cytosol where replication takes place. Host ribosomes now translate both ORFs 1a/1ab into the polyproteins pp1a and pp1ab. These are cleaved into smaller polypeptides and form replication/transcription complexes, probably including additional viral and host proteins. These complexes are membrane-bound and located at virus induced Double Membrane Vesicles (DMV). At these sites the replication of progeny genomes and the production of subgenomic RNAs takes place.

The translated M, E and S proteins accumulate at the ER-Golgi intermediate compartment, as well as the synthesized and encapsidated genomes. When the precursor viral envelope and the nucleocapsid meet new virus particle bud into the ERGIC lumen. From there they are transported in vesicles along the exocytic pathway to the cell membrane and released[43, 128, 154].

3.2 Severe acute respiratory syndrome

Two human coronaviruses, HCoV-229E and HCoV-OC43, are known to be a major cause of mild infections of the upper respiratory tract in winter time, described as the common cold[99]. In 2002 a new human pathogen emerged in the Guangdong province of the People´s Republic of China. A man from Foshan was the first diagnosed patient with an infectious atypical pneumonia (IAP) which the World Health Organization (WHO) later on named *Severe Acute Respiratory Syndrome* (SARS)[187]. The disease was transmitted by droplets as well as by close contact and began to spread to other geographic regions[187]. After having been introduced to the Hong Kong area this pathogen rapidly reached out into 37 different countries all over the world. Finally after the setup of strict travel restrictions and quarantine measures the pandemic could be stopped in July of 2003[186]. SARS was characterized by pyrexia, myalgia, dyspnea and lymphopenia and many patients developed a pneumonia with progressive respiratory failure[111]. At the end of this pandemic the WHO counted 8439 reported cases with 812 having a fatal outcome (~10%)[167].

Early 2003 three independent research groups reported that SARS was caused by a novel human coronavirus (SARS-CoV)[42, 47, 78]. In the same year of 2003, ACE2 has been identified as the cellular receptor for SARS-CoV[92].

As there was no human coronavirus closely related to SARS-CoV the research community was highly interested where this virus had originated from. As wildlife animals were suspected[187], animal traders and workers of meat markets, as well as the animal stocks, in Guangdong were tested for SARS-CoV seroprevalence. With a positive rate of 13% they exceeded the rates of a control group of health workers which were in close contact to SARS patients (<3%). The highest seroprevalence was found in traders of civet cats with up to 72%[183]. A sampling by PCR of animals traded at these markets revealed SARS-like coronaviruses (SL-CoV) almost identical to SARS-CoV present in Himalayan palm civets (*paguma larvata*) and racoon dogs (*Nyctereutes*

procyonoides)[60]. Subsequent studies showed that farmed civet cats did not have any antibodies against SARS-CoV[158], also sequence analysis demonstrated that SL-CoVs in civet cats undergo the same rapid evolutionary change as seen in the human population[144]. These findings pointed to another host species under wild living animals as original hosts, therefore a broad sampling study was conducted including 127 bats, 60 rodents and 11 monkeys. In this study out of 127 bats 29 were tested positive for coronaviruses by PCR and from 14 samples the spike protein cDNA sequence could be isolated. The data suggested the identification of a novel coronavirus related to SARS-CoV with sequence similarity values of 88% on nucleotide and 93% on amino acid level, with some minor differences in the composition of two ORFs, from the Chinese horseshoe bat (*Rhinolophus sinicus*)[87]. A closer look of antibodies against the nucleocapsid protein of this new bat SARS-like coronavirus (bat-SARS-CoV) showed that 84% of the tested sera were positive. Also an alphacoronavirus distantly related to HCoV-229E (79% nucleotide identity) was found in individuals of this bat species.
These and additional findings led to two possible paradigms for the cross-species transmission of SL-CoVs. First, a coronavirus was transmitted from bats to palm civets where it acquired the necessary changes in the spike protein to be able to infect humans. Or second, direct transmission of a bat-SARS-CoV to humans where the spike protein adapted to the human receptor and was then transmitted by close contact to the captive civet cats[59] (figure 7).

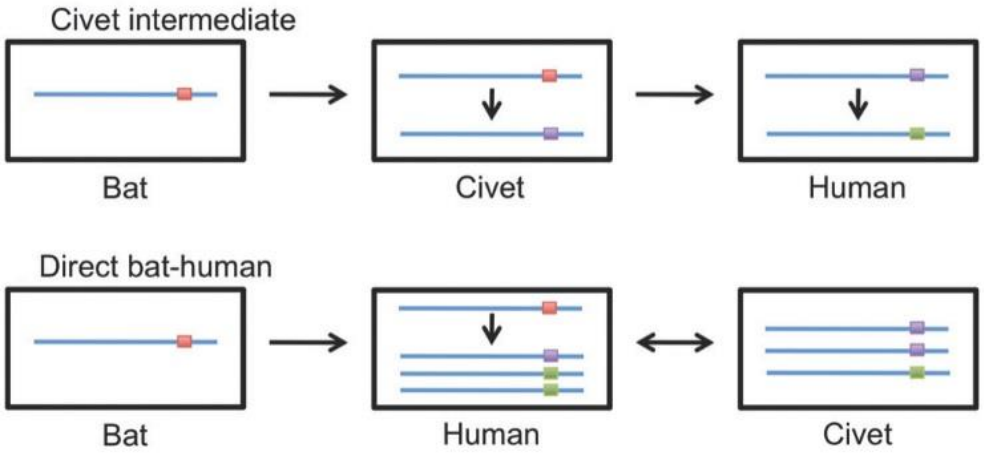

Figure 7: SARS-CoV cross-species transmission
Blue lines representing the spike protein sequence, small boxes the respective RBD domain. Red box indicates a RBD adapted to the bat receptor, purple adapted to civet ACE2 and green to human ACE2.

3.3 Bat as host for emerging diseases

Bats are members of the Order *Chiroptera* which means “hand winged”. From the more than 4,600 known species of mammals about 925 are bats (~20%). They are divided into the suborders Megachiroptera (166 species) and Microchiroptera (759 species)[110]. Probably more than 50 million years old[66], this taxa has been relative stable ever since[148]. Originated on the ancient continent of Laurasia they are today found on every continent except Antarctica[148]. They comprise the only mammalian species that are able of self-powered flight. They travel great distances for their daily food, consisting of fruits, nectar, pollen, insects, small mammals or reptiles, fish and even blood in some cases. Many species migrate during seasons and some *Mexican free-tailed bats* (*Tadarida brasiliensis mexicana*) were found to travel even 1300 km from Mexico to their hibernating sites in Texas[26]. Another feature of members of the families *Vespertilionidae* and *Rhinolophidea* is their ability to reduce their metabolic activity for short and extensive periods of time, respectively known as torpor or hibernation[98]. Overall bats are characterized by extreme longevity and can live up to 35 years, which is not in accordance with the known paradigm for mammals that correlates life expectancy to the ratio between metabolic rate and bodyweight[3].

In general bats are very social and often there is more than one species found at a roost and populations of several million individuals at one site have been reported[28, 102]. Populations of such a size are typically panmictic, as there are no restrictions in mating partners. Some other, for example flying foxes (*Pteropus* spp.), form metapopulations that are spatially separated but interact with each other. At roosts in caves, the density of animals has been reported to be as high as 300 bats per m^2 [28].

A wide range of viruses could be identified in bats[16, 40, 41, 83, 100, 104, 168] and they are reservoir host for several important human pathogens. Especially lyssaviruses have a tight relationship with bats as 10 out of 11 genotypes have been isolated from bats and there is strong phylogenetic evidence that the remaining carnivore rabies virus emerged from a host switch of a bat lyssavirus[5]. Reports of transmission of lyssavirus to other animals are frequent but besides pet animals like especially dogs, humans are very rarely effected by this threat. From 55.000 people die each year of rabies, most of the time unvaccinated dogs are causing the transmission.

Another important group of human pathogens with a host reservoir in bats is classified within the family *Filoviridae,* which comprise the genera *Marburgvirus* and *Ebolavirus*.

Five genetically distinct members of the genus *Ebolavirus* have been identified so far, all of them inducing a disease designated Ebola Haemorrhagic Fever (EHF): *Zaire ebolavirus*, *Sudan ebolavirus*, *Côte d´Ivoire ebolavirus*, *Bundibugyo ebolavirus* and *Reston ebolavirus*. The disease, known for more than three decades, has occurred in sporadic outbreaks over the time in Africa with increasing incidence. Ebolavirus constitutes an important thread to humans as case fatality rates are up to 90 % and neither a vaccine nor an effective treatment are available.

For a long time a connection to bats had been suspected, but in 1996 infection studies demonstrated that bats can serve as hosts for ebolaviruses[146]. Later on studies of antibody prevalence and search for virus-specific nucleic acid provided almost conclusive evidence that bats are indeed a filovirus reservoir[90, 116]. Finally in 2009, Marburg virus was isolated from Egyptian fruit bats[155]. Besides the old world bats found in Africa, there is evidence that filoviruses are endemic in Asian bats as well[185]. Transmission can happen by bites or the handling and consumption of meat. As so-called bushmeat, wildlife animals are still one of the major sources of protein-rich diet in many parts of the world.

In 1994 an outbreak of a novel paramyxovirus was reported in Australia. This virus infected horses and was transmitted to humans[106 137]. Two years later a close relative of this virus, now designated as Hendra virus, was identified in Australian flying foxes[182] and successfully isolated in 2000[63]. From 1994 to 2011 there were 31 reported spillover of that virus, affecting 66 horses and 7 human cases, 4 of which have died. This seems to happen in an even increasing number as for 2011 alone 17 new spillover events have been reported[142]. Part of the pathology in both horses and humans is a severe infection of the respiratory tract as well as neurologic symptoms in some cases. Direct transmission of Hendra virus from bats to humans has not been reported so far, only infection after contact with infected horses.

Just a few years later in 1998, there was an outbreak of another novel infectious pathogen on pig farms in Malaysia, causing severe respiratory and neurologic symptoms in the affected animals and resulted in the culling of millions of pigs. Additionally 257 human patients were reported of which 105 did not survive [24, 56]. As in the case of Hendra virus infection, the respiratory and the neurologic system was affected. A new paramyxovirus was isolated from human samples and designated Nipah virus [24]. Two years later it was identified in bats [180] and 2002 isolated for the first time directly [25] Since then Nipah virus has been identified in several species of flying

foxes in India, Thailand, Cambodia and Indonesia [46, 120, 126, 163]. Genetic analysis of Hendra and Nipah virus made clear that both pathogens are closely related and belong to a different genus than all other known paramyxoviruses, which is now designated *Henipavirus* [164].

The latest interesting finding with respect to a possible threat for human health may be the report of a complete novel influenza A strain in bats of Guatemala [153].

Since the SARS epidemic and the emergence of henipaviruses virologists worldwide had a closer look at bats. Today we know of more than 80 virus species in bats and it appears that the number is increasing every month. This also led to the identification of several new coronaviruses. Alphacoronaviruses were found in bats almost on every continent except for Australia and betacoronaviruses on the African and Eurasian continent (figure 8).

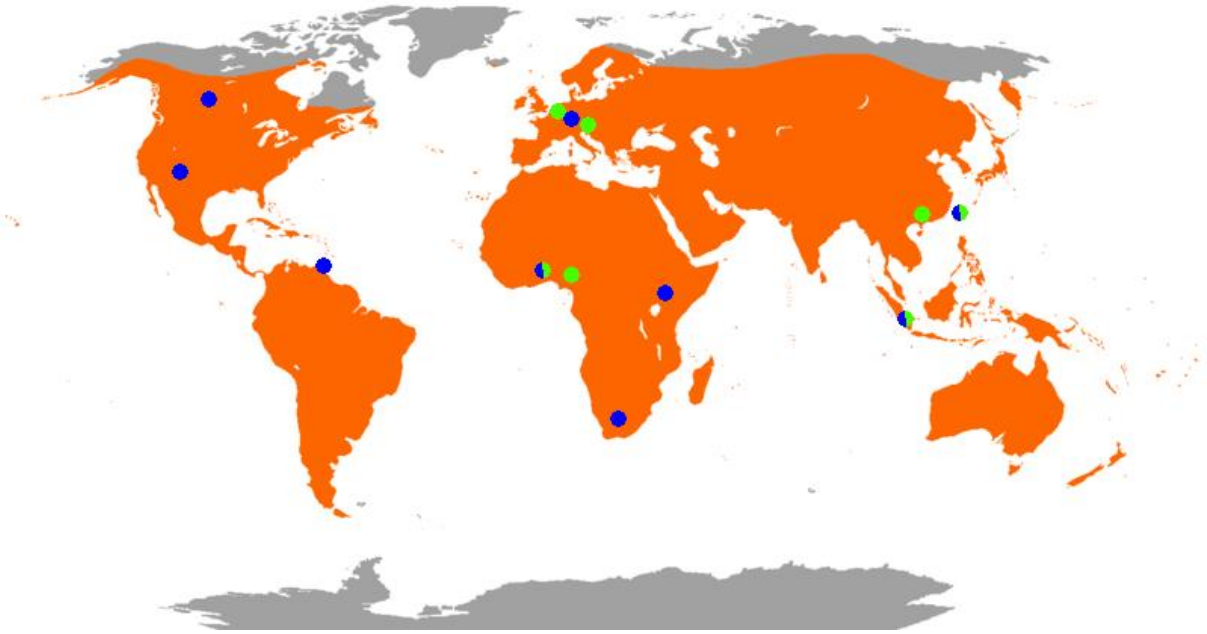

Figure 8: Distribution of bat cornaviruses
Regions colored in orange are habitat of bats. Blue dot marks sample site where alphacoronavirusess where found, green for betacoronaviruses, half blue/green species of both genera [2, 17, 23, 36, 40, 52, 54]

3.4 Pseudotyping the *Vesicular Stomatitis Virus*

The *Vesicular Stomatitis Virus* (VSV) belongs to the family *Rhabdoviridae*, genus *Vesiculovirus*. Its genome is non-segmented, single-stranded RNA in negative orientation and the particles are enveloped. It infects a broad range of animals, like cattle, horses and swine. *In vitro,* almost every mammalian cell can be infected by this virus and it usually grows to very high titer. There are 5 structural proteins, nucleoprotein (N), large protein (L), phosphoprotein (P), matrix protein (M) and the glycoprotein (G). The L and P proteins form the RNA-dependent RNA polymerase whereas the G protein mediates the fusion with the endosomal membrane. Unlike coronaviruses the assembly and budding of VSV happens at the plasma membrane.

To study the processes of viral attachment and fusion laboratories have to maintain very high safety levels if they want to work on viruses like *Ebola Virus*, *Human Immunodeficiency Virus* or SARS-CoV. As there is no vaccine or effective treatment available, to work with these viruses represents a very high risk for the researcher and the human population. There are growing numbers of alternatives to investigate protein-protein interaction or other molecular processes at protein level and the establishment of reverse genetic systems opens up even further possibilities[166]. Viruses are very effective in ways of genome organization and viral architecture. For most of them the structural proteins that build up the viral particle are indispensable. Sometimes it is possible to delete essential genes in the DNA containing the viral genome and substituting them *in trans* by transfecting the host cell. In this way functional particles can assemble but if they are infecting a non-transfected cell the viral replication cannot proceed. This is called a single cycle infection.

The ability of VSV to infect certain cells relies completely on its glycoprotein G which has to be at the plasma membrane to get incorporated into the virion. If this protein is not at hand other membrane proteins get incorporated instead [132]. This opens up the possibility to create VSV particles which do not harbor the VSV glycoprotein but the fusion proteins of other enveloped viruses, a process called pseudotyping. As this viral particle only possess a genome without any viral fusion protein they are only infectious for one round of replication and can be handled at lower safety standards.

To study infection by VSV, there are specific antibodies against the viral proteins available. But the reverse genetic system offers even better possibilities. As the G protein gets eliminated from the genome of VSV for pseudotyping there is now space to insert a gene of interest. For VSV there exist cDNA clones which instead of the G

protein have a green fluorescent protein (GFP) or luciferase gene inserted. After pseudotyping this virus has the tropism of the *in trans* substituted viral fusion protein and infection can be detected by either GFP or luciferase expression.

It has been shown before that VSV can be pseudotyped with a range of different fusion proteins like paramyxovirus, filovirus, arenavirus fusion proteins [65] [49] [50] and also coronavirus spike proteins have been used successfully [48].

4 Material

4.1 Cell lines

Table 2: Immortalised cell lines

Identifier	Species	Tissue	Growth medium
VeroE6	*Chlorocebus sp.*	Kidney	DMEM + 5% FCS
BHK-21	*Mesocricetus auratus*	Kidney	EMEM + 10% FCS
HEK-293T	*Homo sapiens*	Kidney	DMEM + 10% FCS
HeLa	*Homo sapiens*	Cervical cancer	EMEM + 2.5% FCS
RlKd	*Rhinolophus landerii*	Kidney	DMEM + 10% FCS
RhiLu-1.1	*Rhinolophus alcyone*	Lung	DMEM + 10% FCS
RhiNi1.2	*Rhinolophus alcyone*	Kidney	DMEM + 10% FCS
RhiBrain-4p	*Rhinolophus alcyone*	Brain	DMEM + 10% FCS
RhiEuLu	*Rhinolophus euryale*	Lung	DMEM + 10% FCS
RhiFeLu	*Rhinolophus ferrumequinum*	Lung	DMEM + 10% FCS
PipNi-3	*Pipistrellus pipistrellus*	Kidney	DMEM + 10% FCS
PipNi-4	*Pipistrellus pipistrellus*	Kidney	DMEM + 10% FCS
MyDauDa-46	*Myotis daubentonii*	Intestine	DMEM + 10% FCS
MyDauLu-47	*Myotis daubentonii*	Lung	DMEM + 10% FCS
MyDauBrain-48	*Myotis daubentonii*	Brain	DMEM + 10% FCS
MyDauBrain-48B	*Myotis daubentonii*	Brain	DMEM + 10% FCS
HipEm-5	*Hipposideros caffer*	Embryonic	DMEM + 10% FCS
HipEm-28	*Hipposideros caffer ruber*	Embryonic	DMEM + 10% FCS
HipaLu-24	*Hipposideros abae*	Lung	DMEM + 10% FCS
HipaLu-27	*Hipposideros abae*	Lung	DMEM + 10% FCS
EidNi-41	*Eidolon helvum*	Kidney	DMEM + 10% FCS
EidLu-43	*Eidolon helvum*	Lung	DMEM + 10% FCS
RoEnd-4	*Rousettus aegyptiacus*	Endometrium	DMEM + 10% FCS
RoNi-7	*Rousettus aegyptiacus*	Kidney	DMEM + 10% FCS
EpoNi-22.3	*Epomophorus spp.*	Kidney	DMEM + 10% FCS

HypLu-2	*Hypsignathus monstrosus*	Lung	DMEM + 10% FCS
HypLu-45	*Hypsignathus monstrosus*	Lung	DMEM + 10% FCS
HypNi-1	*Hypsignathus monstrosus*	Kidney	DMEM + 10% FCS
HypNi-21	*Hypsignathus monstrosus*	Kidney	DMEM + 10% FCS
Tb1Lu	*Tadaria brasiliensis*	Lung	DMEM + 10% FCS

All bat derived cell lines described in table 2 were provided by Prof. Dr. C. Drosten and Dr. Marcel Müller, University Hospital Bonn. They have been transformed by the large T antigen of *Simian Virus 40.* Experiments were conducted with cells passaged less than 30 times.

4.2 Cell culture media

4.2.1 DMEM (Dulbecco´s Minimal Essential Medium), pH 6.9

DMEM powder	13.53 g/l	GIBCO/Invitrogen, Karlsruhe
$NaHCO_3$	2.20 g/l	Merck, Darmstadt

4.2.2 EMEM (Eagle´s Minimal Essential Medium), pH 7.0

EMEM powder	9.60 g/l	GIBCO/Invitrogen, Karlsruhe
$NaHCO_3$	2.20 g/l	Merck, Darmstadt

4.2.3 Freezing Medium

DMEM / EMEM		
Fetal calf serum	10%	Biochrom, Hamburg
Glycerol (sterile)	10%	AppliChem, Darmstadt

4.2.4 Trypsin/EDTA

NaCl	8.00 g/l
KCl	0.20 g/l
Na_2HPO_4 x 12 H_2O	2.31 g/l
KH_2HPO_4 x 2 H_2O	0.20 g/l
$CaCl_2$	0.13 g/l
$MgSO_4$ x 7 H_2O	1.10 g/l

Trypsin (3 U/mg)	1.25 g/l
EDTA	1.25 g/l
Streptomycin	0.05 g/l
Penicillin	0.06 g/l

4.3 Bacteria media

4.3.1 Luria-Bertani (LB) media

Tryptone	10 g/l	AppliChem, Darmstadt
NaCl	10 g/l	AppliChem, Darmstadt
Yeast extract	5 g/l	Roth, Karlsruhe

4.3.2 LB agar

Tryptone	10 g/l	AppliChem, Darmstadt
NaCl	10 g/l	AppliChem, Darmstadt
Yeast extract	5 g/l	Roth, Karlsruhe
Agar Agar	20 g/l	Roth, Karlsruhe

4.4 Buffers and solutions

4.4.1 Anode buffer I, pH 9.0

Tris 1 M	300 ml/l	Roth, Karlsruhe
Ethanol	200 ml/l	AppliChem, Darmstadt
adjust pH with KCl		

4.4.2 Anode buffer II, pH 7.4

Tris 1 M	25 ml/l	Roth, Karlsruhe
Ethanol	200 ml/l	AppliChem, Darmstadt
adjust pH with HCl		

4.4.3 Cathode buffer, pH 9.0

Tris 1 M	25 ml/l	Roth, Karlsruhe

Aminocaproic acid	5.25 ml/l	Sigma-Aldrich, München
Ethanol	200 ml/l	AppliChem, Darmstadt
adjust pH with HCl		

4.4.4 DAPI staining solution

Ethanol 100%		AppliChem, Darmstadt
4′,6-Diamidin-2-phenylindol (**DAPI**)	1 mg/l	Sigma-Aldrich, München

4.4.5 Ethidium bromide staining solution

TAE buffer		
Ethidium bromide	10 g/l	Sigma-Aldrich, München

4.4.6 Mowiol

Mowiol 4-88	120 g/l	Calbiochem, Heidelberg
Glycerol	300 g/l	Roth, Karlsruhe
DABCO	25 g/l	Sigma-Aldrich, München
Tris/HCl	120 mM	AppliChem, Darmstadt

4.4.7 Paraformaldehyde (PFA), pH 7.4

PBSM		
Paraformaldehyde	30 g/l	AppliChem, Darmstadt

4.4.8 Phosphate buffered saline (PBS), pH 7.5

NaCl	8.00 g/l	AppliChem, Darmstadt
KCl	0.20 g/l	AppliChem, Darmstadt
Na_2HPO_4	1.15 g/l	Merck, Darmstadt
KH_2PO_4	0.20 g/l	Merck, Darmstadt
$MgCl_2$ x 6 H_2O	0.10 g/l	Merck, Darmstadt
$CaCl_2$ x 2 H_2O	0.13 g/l	Merck, Darmstadt

4.4.9 Phosphate buffered saline, minus (PBSM), pH 7.5

NaCl	8.00 g/l	AppliChem, Darmstadt
KCl	0.20 g/l	AppliChem, Darmstadt
Na_2HPO_4	1.15 g/l	Merck, Darmstadt
KH_2PO_4	0.20 g/l	Merck, Darmstadt

4.5 Bacteria

Escherichia coli (E.coli) MRF´ XL-1 blue	Stratagen, La Jolla USA

4.6 Plasmids

4.6.1 pCG1

This plasmid was originally obtained from R. Cattaneo (Mayo Clinic College of Medicine, Rochester, Minnesota, USA). It contains an intron from the rabbit β–tubulin gene which acts as bait for cellular spliceosomes, as well as an ampicillin resistance gene for selection in bacterial cultures.

4.6.2 pCG1-Fc

A derivate of the pCG1 plasmid with the open reading frame of the Fc fragment of human immunoglobulin G inserted at the *SphI* restriction site of the Multiple Cloning Site (MCS). When a coding sequence of a protein is inserted into the MCS in frame with the Fc fragment sequence and without STOP-codon sequences, a chimeric protein is expressed consisting of Fc attached to the protein of choice. Via the C-terminal Fc tag, these proteins can be easily detected by anti-human IgG antibodies. This also allows the purification by FPLC using immobilised protein A columns.

4.6.3 pCG1-Fc-ATG

This is a pCG1-Fc plasmid with an in frame ATG-codon in front of the Fc sequence, which allows to express the Fc tag alone.

4.6.4 pCG1-Fra1-S

This is a pCG1 plasmid with the SARS-CoV spike from the Frankfurt-1 isolate[150] inserted into the MCS. The sequence of this is found in GenBank under the accession number AAP33697.1.

4.6.5 pCG1-Fra1-S1-Fc

This is a pCG1-Fc plasmid with the S1 domain (amino acid 1-667) of the Frankfurt-1 SARS-CoV spike protein inserted into the MCS.

4.6.6 pCG1-Fra1-Sred

This is a pCG1 plasmid with the Frankfurt-1 SARS-CoV spike protein inserted into the MCS. The spike protein is C-terminally connected to a DsRed protein separated by a linker sequence. The exact sequence is included in the supplement.

4.6.7 pCG1-Bg08-S

This is a pCG1 plasmid with the spike protein of a bat SARS-like CoV inserted into its MCS. The Bg08 spike protein was isolated from feces of a *Rhinolophus blasii* bat in Bulgaria. Sequence analysis confirmed that this virus belongs to the betacoronaviruses. It was kindly provided by Prof. Dr. Christian Drosten, University Hospital Bonn, Germany.

4.6.8 pCG1-Bg08-S1-Fc

This is a pCG1-Fc plasmid with the S1 domain (amino acid 1-672) of the Bg08 SARS-like CoV spike protein inserted into the MCS.

4.6.9 pCG1-Bg08-Sred

This is a pCG1 plasmid with the coding sequence of the Bg08 SARS-like CoV spike protein inserted into the MCS. The spike is C-terminally connected to a DsRed protein separated by a linker sequence. The exact sequence is included in the supplement.

4.6.10 pCG1-BB9904-S

This is a pCG1 plasmid with the coding sequence of the spike protein of a bat SARS-like CoV inserted into its MCS. BB904 was isolated from feces of a *Rhinolophus alcyone* bat in Spain. By sequencing it was confirmed that this virus belongs to the

betacoronaviruses. It was kindly provided by Prof. Dr. Christian Drosten and Dr. Jan Felix Drexler, University Hospital Bonn, Germany.

4.6.11 pCG1-BB9904-Sred

This is a pCG1 plasmid with the BB9904 SARS-like CoV spike protein inserted into the MCS. The spike protein C-terminally attached to a DsRed protein separated by a linker sequence. The exact sequence is included in the supplement.

4.6.12 pCG1-Rp3-S1-Fc

This is a pCG1-Fc plasmid with the S1 (amino acids 1-653) domain of a spike protein from a bat SARS-like CoV inserted into its MCS. Rp3 is a bat SARS-like CoV found in *Rhinolophus pearsonii* feces in South-East China[93]. It has the highest similarity to SARS CoV of all known bat CoV (GenBank: AAZ67052.1). The Rp3 spike protein was kindly provided by Prof. Ph.D Hongkui Deng, Peking University, China.

4.6.13 pCG1-hACE2-GFP

This is a pCG1 plasmid with a human ACE2 protein sequence inserted into its MCS. ACE2 is C-terminally attached to a GFP protein. The original human ACE2 protein was kindly provided by Prof. Dr. Eric Snijder, Leiden University Medical Center, Netherlands.

4.6.14 pCG1-hAPN-GFP

This is a pCG1 plasmid with the coding sequence of a human APN inserted into its MCS. APN is N-terminally attached to a GFP protein. This construct was kindly provided by Dr. Christel Schwegmann-Weßels, University of Veterinary Medicine Hannover, Germany.

4.6.15 pCG1-hDPP4-GFP

This is a pCG1 plasmid with the coding sequence of a human DPP4 inserted into its MCS. DPP4 is N-terminally attached to a GFP protein. The original DPP4 protein was kindly provided by Prof. Dr. Hassan Naim, University of Veterinary Medicine Hannover, Germany.

4.6.16 pCG1-RNACE2-GFP

This is a pCG1 plasmid with the coding sequence of a bat ACE2 protein inserted into its MCS. This ACE2 is C-terminally attached to a GFP protein. It was isolated from the

RlKd (*Rhinolophus landeri*) cell line by RT-PCR. The exact sequence is included in the supplement.

4.6.17 pCG1-RLACE2-GFP

This is a pCG1 plasmid with the coding sequence of a bat ACE2 protein inserted into its MCS. This ACE2 is C-terminally attached to a GFP protein. It was isolated from the RhiLu1.1 (*Rhinolophus alcyone*) cell line by RT-PCR. The exact sequence is included in supplement.

4.6.18 pCG1-bDPP4-GFP

This is a pCG1 plasmid with the coding sequence of a bat DPP4 protein inserted into its MCS. At the N-terminus this DPP4 is connected to a GFP protein. It was isolated from the RhiLu1.1 (*Rhinolophus euryale*) cell line by RT-PCR. The exact sequence of bDPP4 and bDPP4-GFP is included in the supplement.

4.7 Enzymes

4.7.1 Restriction enzymes

BamHI	Fermentas, St. Leon-Rot
PacI	Fermentas, St. Leon-Rot
SalI	Fermentas, St. Leon-Rot
XbaI	Fermentas, St. Leon-Rot
XhoI	Fermentas, St. Leon-Rot

4.7.2 Other enzymes

Phusion High Fidelity polymerase	Fermentas, St. Leon-Rot
Taq polymerase	Fermentas, St. Leon-Rot
T4 DNA ligase	Fermentas, St. Leon-Rot

4.8 Antibodies

The following table 3 lists the secondary antibodies used in immunofluorescence (IF) or Western Blot (WB).

Table 3: Antibodies

Name	Dilution	Usage	Company
Goat anti-human Alexa-Fluor 488	1:1000	IF	Invitrogen, Darmstadt
Goat anti-human Cy3	1:500	IF	Sigma-Aldrich, Munich
Goat anti-human IgG, Horseraddish peroxidase	1:5000	WB	Sigma-Aldrich, Munich

4.9 Kits

QIAquick PCR Purification Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
NucleoBond Xtra Midi Kit	Macherey-Nagel, Düren
RNeasy Mini Kit	Qiagen, Hilden
BCA Protein Assay Kit	Thermo-Scientific, Dreieich
Luciferase Assay System	Promega, Mannheim

4.10 Chemicals

1,4-Dithiotreitol (DTT)	Roth, Karlsruhe
2-Mercaptoethanol	FLUKA, Basel
Acrylamide solution 30% (*Rotiphorese Gel 30*)	Roth, Karlsruhe
Agar Agar	Roth, Karlsruhe
Agarose	Biozym, Hess. Oldendorf
Aminocapronic acid	Sigma-Aldrich, Munich
Ammonium persulfate (APS)	Bio-Rad, Munich
Boric acid	Roth, Karlsruhe
Calcium chloride	Roth, Karlsruhe

DEPC treated water	Roth, Karlsruhe
Disodium hydrogen phosphate	Roth, Karlsruhe
dATP	Fermentas, St. Leon.Rot
dCTP	Fermentas, St. Leon.Rot
dGTP	Fermentas, St. Leon.Rot
dTTP	Fermentas, St. Leon.Rot
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe
Acetic acid	Roth, Karlsruhe
Ethanol	Merck, Darmstadt
Ethidiumbromide	Sigma-Aldrich, Munich
Fetale calve serum (FCS)	Biochrom AG, Hamburg
Glucose	Roth, Karlsruhe
Glycerine	Roth, Karlsruhe
Glycin	Roth, Karlsruhe
Hydrochloric acid	Roth, Karlsruhe
HEPES	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe
Magnesium chloride	Roth, Karlsruhe
Magnesium sulfate	Roth, Karlsruhe
Methanol	Roth, Karlsruhe
Mowiol	Calbiochem, Heidelberg
N,N,N’,N’-Tetramethylene diamine (TEMED)	Roth, Karlsruhe
Non-essential aminoacids	Biochrom AG, Hamburg
Paraformaldehyde	FLUKA, Basel
Polyethylenimine	Roth, Karlsruhe
Potassium chloride	Roth, Karlsruhe
Potassium dihydrogen phosphate	Roth, Karlsruhe
Sodium acetate	Merck, darmstadt
Sodium chloride	Roth, Karlsruhe
Sodium desoxycholate	Roth, Karlsruhe
Sodium phoshate	Roth, Karlsruhe
Sodium dihydrogen phosphate	Roth, Karlsruhe
Sodiumdodecylsulfat (SDS)	Roth, Karlsruhe

Sodium hydrogen phosphate	Roth, Karlsruhe
Sodium hydroxide	Roth, Karlsruhe
Tris-Hydroxymethylaminomethan (TRIS)	Roth, Karlsruhe
Trypton	Roth, Karlsruhe
Tween 20	Roth, Karlsruhe
Yeast extract	Roth, Karlsruhe

4.11 Other substances

Blocking reagence	Roche, Mannheim
Super Signal West Dura Extended Duration Substrate	Pierce, Rockford (USA)
Super Signal West Femto Extended Sensitivity Substrate	Pierce, Rockford (USA)
Spectra multicolour high range protein ladder (300 kDa)	Fermentas, St. Leon-Rot
Gene Ruler 1 kb DNA Ladder plus	Fermentas, St. Leon-Rot
Lipofectamine 2000 reagent	Invitrogen, Darmstadt

4.12 Equipment

4.12.1 Agarose gel electrophoresis

Electrophoresis Box, Gel Mold, Gel Comb	Keutz, Reiskirchen
Microwave MWS 2820	Bauknecht, Schorndorf
UV-Transluminator	UVP, Upland, (USA)
Swiveling table	Keutz, Reiskirchen
Power Supply	Bio-rad, München
Eppendorf BioPhotometer Plus	Eppendorf AG, Hamburg

4.12.2 Bacteria culture

Petri dishes, 100mm	Greiner, Nürtingen
Erlenmeyer flask, 100ml, 300ml, 500ml	Jürgens, Hannover

Shaking incubator Type 3033	GFL, Burgwedel
Incubator Type B16	Heraeus, Osterode

4.12.3 Cell culture

Tissue culture flasks 75 cm^2	Greiner, Nürtingen
96-well plates (flat bottom)	Costar Corning, Sigmar-Aldrich, Munich
24-well plates	Greiner, Nürtingen
6-well plates	Greiner, Nürtingen
CO_2 incubator	Heraeus, Hanau
Swiveling table	Keutz, Reiskirchen
Coverslips	Roth, Karlsruhe
Microscope slide	Roth, Karlsruhe

4.12.4 Centrifuges

Microcentrifuge 5415 D	Eppendorf, Hamburg
Megafuge 1,0R	Heraeus, Hanau
Centrifuge 5417C/R	Eppendorf, Hamburg

4.12.5 Fast Protein Liquid Chromatography

Membrane Filter 0.8 µm and 0.45 µm, 50 mm in diameter	Landgraf Laborsysteme, Burgwedel
Hitrap Protein A HP 1 ml and 5 ml	GE Healthcare, Freiburg
FPLC	GE Healthcare, Freiburg

4.12.6 Magnetic stirrer

Magnetic stirrer, RCT basic	IKA Labortechnik, Staufen

4.12.7 Microscope

Eclipse *Ti*	Nikon, Düsseldorf

NIS Elements Imaging Software (64bit, 3.22.11; Build 728)	Nikon, Düsseldorf

4.12.8 PCR

Prismus 25/96 Thermocycler	MWG Biotech, Ebersberg
0.2ml PCR reaction tube	Biozym, Hess. Oldendorf

4.12.9 pH-Meter

pH-Meter	Jürgens, Hess. Oldendorf

4.12.10 Pipettes and pipette helpers

10 µl, 100 µl, 1000 µl	Eppendorf, Hamburg
10 µl, 100 µl SafeSeal-Tips	Biozym, Hess. Oldendorf
1 ml, 2 ml, 5 ml, 10 ml, 20 ml glas pipettes	Jürgens, Hannover
AccuJet Pipette Helper	Brand, Wertheim/Main

4.12.11 Reaction tubes, columns and sterile filters

FP 30/0.2 CA-S sterile filter	Schleicher & Schuell, Dassel
15 ml and 50 ml reaction tubes	Greiner, Nürtingen

4.12.12 Safety cabinettes

NuAire Class II	Nuaire, Plymouth (USA)
Hera Safe	Heraeus, Hanau
NuAire Class II Type A/B3	Nuaire, Plymouth (USA)
KOJAIR KR-130 BW MSC CL II EN12469	KOJAIR, Vilppula, Finland

4.12.13 SDS-PAGE and Semi-dry Western-Blot

Slab Gel chamber	Keutz, Reiskirchen
Filter paper	Schleicher & Schuell, Dassel
Nitrocellulose transfer membrane	Schleicher & Schuell, Dassel

Transfer chamber	Biometra, Analytic Jena, Ober-Ramstadt
ChemiDoc EQ	Bio-rad, München
Quantity One V 4.4.0 (Software)	Bio-rad, München

4.12.14 Vortex

Reax top	Heidolph, Kehlheim
Reax 2000-05-20	Heidolph, Kehlheim

4.12.15 Scales

Electronic analysis scale, Type 1712 MP 8	Sartorius, Göttingen
Sartorius Portable scale Lauda A100	Sartorius, Göttingen

4.12.16 Water bath

Water bath	GFL, Burgwedel

5 Methods

5.1 Cell culture

The cell lines used in this thesis were all cultured at 37°C and 5 % CO_2. The continuous cultures were grown in 75 cm^2 culture flasks (Greiner), in a volume of 10-20 ml of the respective medium. The cell were passaged 1-3 times a week depending on the growth characteristics. For passaging of the cells, the depleted medium was removed and the cells gently washed with 5 ml PBSM. Next, 1-5 ml of trypsin/EDTA was added and the cells incubated for several minutes until all cells had been detached. After that the cells were resuspended in medium and usually in ratios of 1:5 - 1:20.

5.1.1 Mycoplasm test

To test for the presence of eventual mycoplasma contamination, every cell line in culture was stained with DAPI (4´,6´-Diamidino-2-phenylindol) in two weeks intervals. For this purpose, suspended cells were seeded onto coverslips in 24 well plates. After 5 hours the medium was removed and the cells washed with PBSM, before they were incubated with 250 µl DAPI per well for 15 minutes. Then, the DAPI reagent was disposed and the cells washed 2 times with PBSM. The staining was subsequently analysed by laser scanning microscopy. In additional to this test, every two months a mycoplasma-specific PCR was carried out by our lab technician.

5.1.2 Cryoconservation

Cell lines were stored at 80°C while not in culture. Therefore, they were pelleted at 500 g using a centrifuge and subsequently re-suspended in freezing medium, at a density of $1x10^5$ cell per ml. They were then aliquoted in portions of 1 ml in cryo-tubes and slowly frozen.

To take these cell back into culture, the aliquots were thawed quickly at 37°C in a water bath, re-suspended in 10 ml medium and pelleted at 500 g. Afterwards the cells were seeded into the cell culture flasks.

5.1.3 Transfection by lipofectamine

For several experiments we used lipofectamine for transfecting cells, because of the high transfection efficiency and low cytotoxicity of this reagent. In one experiment HeLa cells were transfected for expression of receptor candidates and subsequently incubated with soluble spike proteins. For this purpose, HeLa cells were seeded on coverslips in 24 well plates, $5x10^5$ cells per well in 500 µl. On the next day, the cells were transfected with 1 µg plasmid DNA according to the manufacturer protocol and incubated for additional 12-16 h.

The production of VSV pseudotypes also requires transfection by lipofectamine. Here, $2x10^5$ BHK-21 cells were seeded onto 10 cm dishes in 10 ml medium. On the next day, the cells were transfected with 8 µg plasmid DNA according to the manufacturer´s protocol and 24h later infected with VSV-ΔG-G. By the same parameters, the transfection of BHK-21 for the cell based binding assay was performed.

In the VSV pseudotype assay, BHK-21 cells were transfected to analyse the specific receptor candidates whether they rendered the cells more susceptibility to infection or not. For this purpose, BHK-21 cells were seeded in 96 well plates at a density of $2x10^4$ cells per well and 100 µl medium total. On the following day each well was transfected with 0.1 µg plasmid DNA by lipofectamine according to the manufacturer´s protocol. The infection with the VSV pseudotypes was performed the following day.

5.1.4 Transfection by polyethylenimine

The cell based binding assay required very specific parameters for the transfection, which could only be met by polyethylenimine (PEI) transfection. For this purpose, $5x10^5$ HeLa cells were re-suspended in 500 µl EMEM without FCS. In parallel the transfection mix was prepared, containing 1 µg plasmid DNA and 2.58 µg PEI. Both, DNA and PEI, were diluted in 50 µl each, mixed after 5 min incubation, and incubated for further 20 min. After seeding the 500 µl HeLa cells onto the plate, the transfection mix was added immediately. Then the 24 well plate was incubated in the incubator, at first for 1 h on a swivelling table followed by additional 5 h without shaking. Afterwards the medium was removed and 1 ml fresh EMEM with FCS was added. After an incubation for 16 h, these cells were the used in the cell based binding assay.

5.1.5 Transfection by calcium phosphate precipitation

For the production of soluble spike proteins, large quantities of HEK-293T had to be transfected by calcium phosphate precipitation, as this is a very inexpensive transfection reagent. First, HEK-293T cells were seeded onto forty 10 cm dishes with $1.8x10^5$ cells per ml and 10 ml per dish in total. On the next day the medium was exchanged to 5 ml without any FCS. Then the transfection mix was prepared, containing 18 µg plasmid DNA per dish. For this purpose, 720 µg DNA were diluted in 8 ml distilled water. In a separate tube, a volume of 10 ml 2xHBS buffer and 2 ml $CaCl_2$ (1 M) were mixed. Subsequent both DNA and the reagent were mixed and incubated for 5 min. From this mix 500 µl were given onto the dishes in a dropwise fashion. The cells were then incubated for 12-16 h overnight. On the next day, the medium was exchanged and fresh EMEM with 3 % FCS was added. After additional 24 and 72 h, the supernatant was collected and prepared for FPLC purification.

5.2 Molecular biology

5.2.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is used to amplify nucleic acids. During this work, PCR was used several times at different conditions. The *Phusion* polymerase was used to amplify DNA for cloning purposes as this enzyme offers a very efficient proof reading function, i.e. the frequency of nucleotide exchanges during the replication is low. The *Taq* polymerase lacks such a proof reading capability and was only used for analytic PCRs, like the colony PCR. The following table lists the composition of the reaction mixes and the temperature profiles, for *Phusion* or *Taq* PCRs respectively.

***Phusion* PCR 50 µl reaction mix:**

5x Phusion reaction buffer	10 µl
dNTP (10 mM)	1 µl
Sense primer	2.5 µl
Antisense primer	2.5 µl
Template DNA	0.1 µg
Phusion polymerase	0.5 µl
DEPC treated water	Add to 50 µl

***Taq* PCR reaction mix for 1 sample:**

10x Taq reaction buffer	1.5 µl
$MgCl_2$	1.2µl
dNTP (10 mM)	0.3 µl
Sense primer	0.45 µl
Antisense primer	0.45 µl
Taq polymerase	0.1 µl
DEPC treated water	Add to 15 µl

***Phusion* PCR temperature profile:**

	Temperature (°C)	Time (sec)
	95	60
30 cycles	95	30
	54	30
	72	30 / 1 kb of amplificate
	72	5
	4	pause

***Taq* PCR temperature profile:**

	Temperature (°C)	Time (sec)
	95	60
35 cycles	95	30
	54	30
	72	60 / 1 kb of amplificate
	72	5
	4	pause

5.2.2 PCR purification

The used buffer in the *Phusion* PCR reaction interferes with the buffer of restriction enzymes. Prior to digestion of PCR amplified DNA we applied the QIAquick PCR purification Kit (Qiagen) to remove remaining PCR buffer and enzyme.

5.2.3 Enzymatic DNA digestion

To insert a specific DNA sequence into the MCS of a plasmid, first the desired DNA as well as the plasmid has to be digested by restriction enzymes. We therefore used 5 U restriction enzyme for 1 µg plasmid or 20 U for PCR amplified DNA, in a volume of 50 µl total containing enzyme buffer and DEPC treated water. The reaction mix was incubated overnight at 37°C and the DNA subsequently purified by DNA gel extraction.

5.2.4 Agarose gel electrophoresis

To separate DNA according to its size we used agarose gel electrophoresis. This helps to identify amplified DNA in analytic PCRs as well as to purify it after a DNA digest. For analytic purposes TBE buffer was used to prepare the gels and run them in the electrophoresis chamber, whereas gel extraction requires TAE buffer. Generally, gels with an agarose content of 1-2 % were used and run at 130 V (TBE) or 80 V (TAE) for 30-60 min. To visualise the DNA the gels were incubated for about 5 min in a TAE buffer containing ethidiumbromide (1:10.000). Afterwards DNA could be detected under UV light.

5.2.5 DNA gel extraction

PCR amplified DNA that had to be inserted into a plasmid was separated in an agarose gel using TAE buffer. The DNA was then purified from the agarose using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer instructions.

5.2.6 DNA ligation

To insert a specific DNA sequence into a plasmid, first both DNAs had to be digested with DNA restriction enzymes. These enzymes cut DNA at specific sequence sites and leave overhangs of 1-3 nucleotides which can be used to re-ligate the DNA. We therefore opened up the circular plasmid at its MCS with specific restriction enzymes and used the same later on to digest the PCR-amplified DNA at its ends. When both plasmids are mixed they can be ligated to one circular plasmid using the T4 DNA ligase. A usual reaction mix of a volume of 20 µl contains 5 U ligase as well as insert and plasmid DNA in a ratio of 5:1. The ligation reaction was incubated overnight at 14°C.

5.2.7 Transformation of *Escherichia coli*

To amplify re-ligated DNA plasmids, as well as for replenishing stocks, we transformed them into transformation competent *E.coli* XL1-blue by heat-shock. Hereby an aliquot of bacteria, which was stored at -80°C, was gently thawed on ice for 30 min. Then 5 µl of a ligation mix, or 0.5 µg as for replenishing purposes, was added to the bacteria and further incubated on ice for additional 30 min. The bacteria are then shocked for 45 sec at 42°C in a water bath and afterwards put back on ice for additional 2 min. Then 250 µl LB medium were added and the bacteria incubated for 1 h at 37°C in a shaking incubator. Afterwards they were plated onto LB agar dishes containing selective antibiotics and cultured for 12-18 h at 37°C until colonies were visible.

5.2.8 Colony PCR

To distinguish bacterial colonies of *E.coli* after transformation, whether or not they have incorporated the desired plasmid, an analytic colony PCR was performed. A PCR reaction mix was prepared and aliquoted in PCR tubes, as well as 250 µl LB medium in an Eppendorf tubes. The, we dipped a pipette tip into a bacteria colony and subsequently into the PCR reaction mix and afterwards the LB medium. Both times we pipetted a small volume of the respective liquid to re-suspend some bacteria. The PCR was performed and analysed by agarose gel electrophoresis, where in parallel the Eppendorf tube was incubated at 37°C. When the electrophoresis revealed a specific DNA band, the correlating inoculated LB medium was used to prepare an overnight culture.

5.2.9 Plasmid preparation

To gain large amounts of plasmid DNA *E.coli* overnight cultures were prepared. This is a volume of 100 ml LB medium plus the selective antibiotic inoculated with 100 µl of the 250 µl pre-incubated bacteria from the transformation. This culture was incubated in a shaking incubator at 37°C for 16-18 h. Afterwards the plasmid DNA was extracted from the bacteria using the NucleoBond Xtra Midi Kit according to the manufacturer´s protocol.

5.2.10 DNA concentration measurement

The concentration of DNA was determined by photometric analysis. For this, a 10-25 fold diluted DNA solution was prepared and the absorption measured at a wavelength of 260 nm.

5.2.11 DNA sequencing

After a cloning procedure the identity of the DNA sequences had to be confirmed by sequencing. The sequencing was done by MWG Eurofins.

5.2.12 RT-PCR

Reverse Transcriptase is an enzyme that translates RNA into cDNA, which makes it possible to transcribe back cellular mRNA and insert it into a plasmid. One 10 cm dish was seeded with *Rhinolophus* cells and grown to 90 % confluence. The cells were then detached by trypsin treatment and pelleted at 4°C and 500 g in a centrifuge. From this cell pellet the total RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA synthesized with the Superscript III Reverse Transcriptase, using random hexamers as well as oligo-dT primer. This cDNA was pooled and used in a subsequent PCR with gen specific primer to amplify the gen of interest, which was then inserted into pCG1.

5.3 Protein biochemistry

5.3.1 Production of soluble spike proteins

To analyse the bat cell lines as well as transfected HeLa cell for possible receptor candidates, soluble coronavirus spike proteins were generated. By connecting the S1 domain of the respective CoV spikes to a human IgG fragment, we generated chimeric proteins which are simple to produce and purify. They recognize and bind to the specific receptor proteins and can be detected by anti-human IgG antibodies. For the production large quantities of HEK-293T cells were transfected and the supernatant harvested. In a first step of cleaning, the supernatants were centrifuged for 30 min at 4°C and 2800 g, to pellet cell debris.

5.3.2 Protein purification by Fast Protein Liquid Chromatography

After the first purification by centrifugation, the supernatants were passed through a 0.8 µm and a 0.45 µm filter. The filtered liquid was degassed for 1 h. To apply the supernatants to the FPLC, they had to be filled into so called ´loops´, which withstand the high pressures applied during chromatography. The supernatants were then pressed through columns densely packed with immobilised Protein A, which has a high affinity to human IgG. After the entire volume of the supernatant passed the column it was washed with PBS to clear it from all remaining sample remnants. The bound

protein was now eluted by applying a small volume (10-30 ml) of a low pH buffer (0.1 M sodium citrate, pH 3). The elution was captured and fractionated into 1 ml fractions and the pH normalised by addition of 200 µl of TRIS buffer (pH 9). To identify the exact fractions in which the protein was present, 10 µl of each fraction was separated in SDS-PAGE and detected in a subsequent Western-Blot. Positive fractions were pooled and the protein content measure by BCA assay (Thermo-Scientific) according to the manufacturer protocol. The soluble proteins were stored in aliquots of 50 µl at -20°C.

5.3.3 SDS PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method to separate proteins according to their molecular weight. The used gels consisting of a separating phase with 8 % acrylamide and a stacking gel with a lower concentration[85]. The electrophoresis was performed at 80 V until the proteins accumulated at the boundary of both gel types, afterwards the voltage was increased to 130 V to separate the proteins. Usually it took about 60 min to separate proteins of smaller size and up to 90 min for larger ones.

5.3.4 Western blot

To specifically detect proteins separated by SDS-PAGE they have to be transferred from the polyacrylamide gel onto a membrane, to be accessible to antibodies. For this purpose, we used a nitrocellulose membrane and a semi-dry blotting technique[84]. Gel, membrane and filter paper are arranged into a stack between anode and cathode of the blotting chamber. The arrangement is as follows, starting at anode site: 2 filter paper soaked in anode buffer I, 1 filter paper soaked in anode buffer II, the nitrocellulose membrane activated in water shortly before, the gel, 3 filter paper soaked in cathode buffer. After the assembly and closing the chamber tightly, the transfer was started by applying 300 mA. Usually 15-20 min sufficed to transfer proteins even up to sizes of 300 kDa. After the disassembly of the stack the membrane was washed in PBSM one time and then the still active membrane blocked with blocking reagent (0.5 % in PBSM) overnight at 4°C on a swiveling table. On the next day it was washed three times in PBSM + 0.1 % Tween for 15 min. For detecting the Fc tagged protein, the membrane was now incubated in PBSM containing a goat anti-human IgG antibody

(1:5000) which was coupled to horseradish peroxidase, for 1 h at room temperature. After that the membrane was washed three times in PBSM and a suitable peroxidase substrate was applied. The resulting chemiluminescence was analysed with the ChemiDoc Imager (Bio-Rad).

5.3.5 Immunofluorescence

Potential binding of soluble spike proteins to the receptors was detected by applying soluble spike proteins to the suspected cells, immobilised on coverslips. Therefore the cells, transfected by lipofectamine or not, were grown on coverslips and fixed by paraformaldehyde. After that the coverslips were incubated top-down in 20 nmol of soluble spike protein, in a total volume of 20 µl. After 1 h, the coverslips were washed three times with PBS and then incubated top-down in 20 µl anti-human IgG Alexa Fluor-488 antibody (1:1000). Another 1 h later, the coverslips were again washed three times and embedded in Mowiol. On the next day the coverslips were analysed by using the Nikon *Ti* laser microscope.

5.4 Virological Methods

5.4.1 Pseudotyping of *Vesicular Stomatitis Virus*

To generate VSV pseudotypes with another viral surface protein, cells expressing this protein at their plasma membrane are infected by VSV-ΔG-G and the released pseudotypes collected. For this, BHK-21 cells were seeded onto 10 cm dishes and transfected for expression of one the various spike proteins, or VSV G as positive control. On the day of infection the medium was removed and placed by 2.5 ml fresh medium without FCS. Then, VSV-ΔG-G virus at a Multiplicity of infection (MOI) of 3 was added. BHK-21 cells were inoculated for 1 h at 37°C on a swivelling table. Afterwards, the inoculum was removed and the cells washed one time with medium. This medium was then replaced by 2.5 ml medium containing polyclonal rabbit anti VSV-G serum (1:1000). Again, the cells were incubated for 1 h at 37°C on a swivelling table. Then the medium was removed, the cells washed one time and replaced by 7.5

ml fresh EMEM with 3 % FCS. On the next day, 18-20 h after infection, the supernatant was collected and cleaned from debris by 20 min centrifugation at 4°C and 2800 g. The pseudotypes were stored at 4°C for up to 2 weeks without a significant drop in infectivity.

5.5 Analytic assays

5.5.1 Cell based binding assay

This assay is a variation of an already published method of Chou *et al.* in 2005[22]. Here, the spike protein and the receptor are expressed on two separate cell populations. The receptor population can be either cells like VeroE6, different bat cell lines or HeLa cells transfected with different receptor candidates. On the other hand we have BHK-21 cells transfected for the expression of the different with the different CoV spike proteins. The receptor cells were seeded in 24 well plates at a density of 3-4x10^5 cells per well, and grown to 100 % confluence over the course of two days. On the day of the assay, the cells expressing the spike proteins were washed one time with PBS. Then 3 ml Accutase was added and the dishes incubated for 20 min at 37°C. Afterwards the cells were re-suspended in 30 ml EMEM total and counted. The cells were then pelleted by centrifugation for 5 min at 4°C and 500 g, the medium decanted and the cells re-suspended. The volume of added EMEM was exactly calculated so the suspension had a final cell density of 1x10^6 cell per ml.

The receptor cells were washed one time with PBS and then 500 µl of the BHK-21 cell suspension was given into the wells. This was followed by 4 h incubation at 4°C. After the incubation, the wells were washed 3 times with 1 ml PBS (0.5 M $CaCl_2$). In the end, 250 µl PBS were given into the wells and subsequently photographed under the Nikon laser microscope. For the analysis about 20 % of the total well surface had to be photographed by making 5x5 adjoining single shots with 25 % overlap. The number of bound BHK-21 cells was assessed by focusing on the DsRed expression. The Nikon *Ti* software offers the option to count objects automatically and has two important parameters, fluorescence intensity and surface area of the counted object. The threshold for the fluorescent intensity was set to a lower limit of 400 and an upper limit

of 4095(max). All objects with a surface area equal or higher than 20 nm^2 were counted.

5.5.2 VSV-pseudotype luciferase assay

This assay relies on the luciferase encoded in the recombinant VSV genome. By pseudotyping the particles with different CoV spike proteins we altered their natural receptor tropism which was the criteria to be assessed. For this assay VeroE6, bat cells or BHK-21 cells transfected with receptor candidates were tested for susceptibility. They were seeded in 96 well plates with $2x10^4$ cells per well and grown for 2 days, where the BHK-21 cells were transfected on the first day after seeding. For the infection the medium was removed and 25 µl of the VSV-pseudotypes added on top of the adherent cells. The plates were incubated 1 h at 37°C on a swivelling table. After that, the inoculum was disposed and exchanged by 50 µl fresh medium. On the next day, after 14-16 h, the medium was removed and the well washed one time with PBS. Then 25 µl of the lysis buffer was given into each well and incubated at room temperature for 30 min. After the incubation time 25 µl of the luciferase agent was added on top of the cell lysate and the luciferase activity measured using the ChemoImager.

6 Results

6.1 Expression and purification of soluble spike proteins

To analyse the interaction of SARS-like coronaviruses with host cells, surface proteins S were generated in a soluble form. These soluble spike proteins only contain the presumptive S1-subdomains which are fused to a human IgG-Fc tag at the C-terminal end. For production of these proteins, HEK-293T cells were transfected and the supernatant harvested two times over a period of 72 h. After the harvest the supernatants were clarified by centrifugation as well as filtration and applied to FPLC for purification. A column with immobilised protein A was utilized to capture the proteins via their Fc tag. Purity and a rough estimation of the protein content was analysed by subsequent Western-Blot analysis. The analysis of the purified soluble spike proteins under non-reducing conditions revealed a specific band of about 280 kDa. As the estimated molecular weight of the unglycosylated polypeptide including the Fc-tag is about 120 kDa, this 280 kDa band may represent a glycosylated dimeric form of the produced spike proteins. Under reducing conditions (+DTT), a band of about 140kDa is detectable (figure 9). This result suggests that the purified S1-Fc is a dimer consisting of two monomers connected by disulphide bonds.

The soluble spike proteins purified by FPLC reached concentrations of 0.004-0.016 M. For the detection by immunofluorescence, 20 nmol of the proteins were applied in a total volume of 20 µl. For this assay cells, were grown on coverslips, incubated with the proteins bound S1-Fc was visualised by immunostaining.

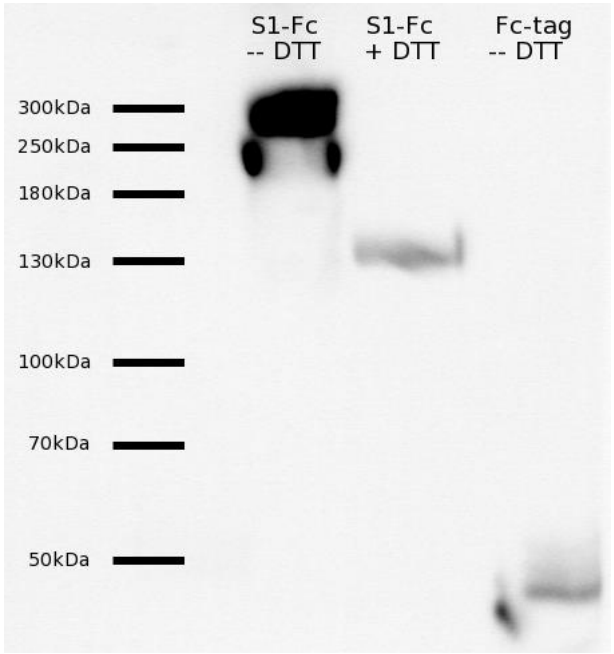

Figure 9: Western-Blot analysis of soluble Fra1-S1-Fc protein
Purified Fra1-S1-Fc was separated by SDS-PAGE in the absence or presence of DTT and analyzed by Western blot. The Fc-tagged proteins were visualized by immunostaining. A sample containing an Fc tag served as a control.

6.2 Binding of soluble spike proteins to human ACE2

VeroE6 cells, which are susceptible to SARS-CoV and express human ACE2, were used to confirm the ability of Fra1-S1-Fc to specifically bind to hACE2. The cells were grown on coverslips and incubated with 20 nmol Fra1-S1-Fc and subsequently stained by Alexa Fluor-488 coupled anti-human IgG antibody. Binding of Fra1-S1-Fc was clearly identified (figure 10). The pattern of binding was unevenly distributed over the cell surface and resembled the hACE2 distribution.

Co-localisation of both proteins was demonstrated when hACE2-GFP was heterologous expressed in HeLa cells and bound by Fra-S1-Fc (figure 11).

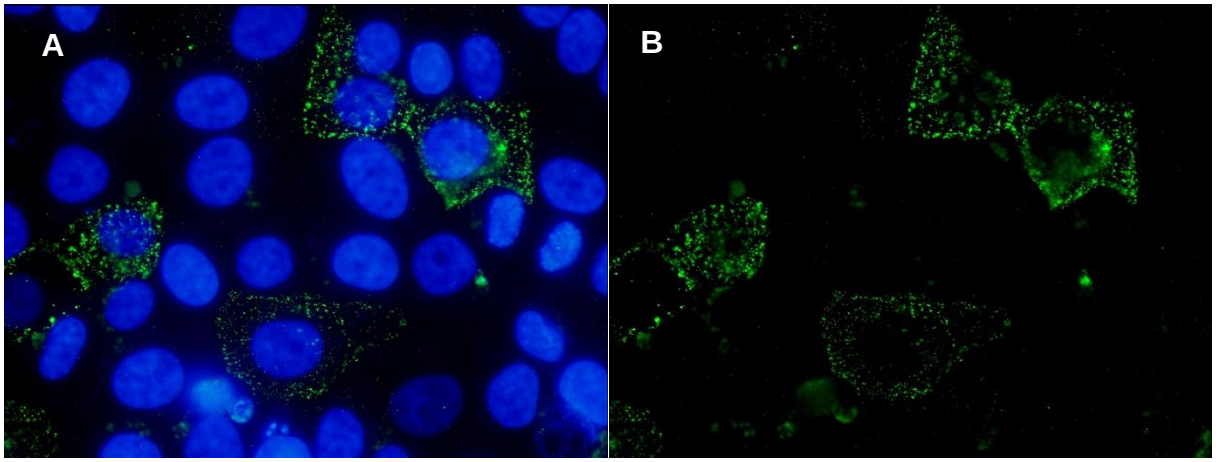

Figure 10: Binding of soluble Fra1-S1-Fc protein to VeroE6 cells
VeroE6 cells were incubated with 20 nmol of Fc-tagged S1 and stained with Alexa Fluor-488 anti-human IgG antibody (A and B). Nuclei were visualized by DAPI staining (A).

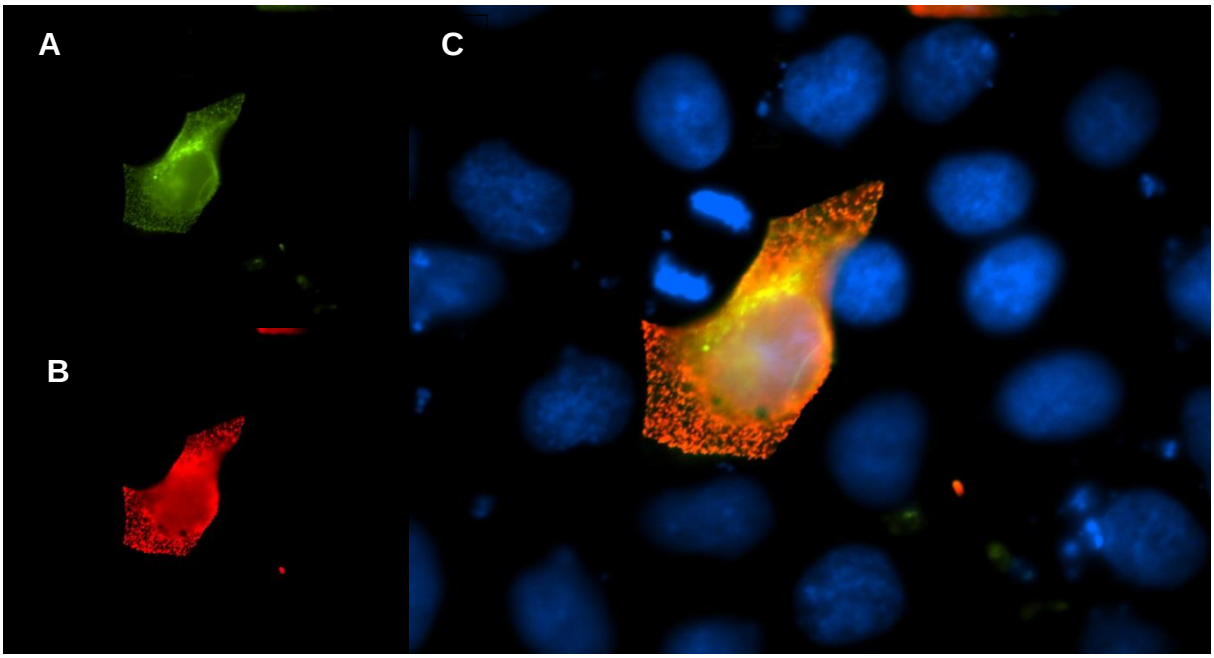

Figure 11: Binding of soluble Fra-S1-Fc protein to HeLa cells transfected for expression of hACE2-GFP
HeLa cells were transfected for transient expression of hACE2-GFP and incubated with 20 nmol Fra2-S1-Fc. Bound coronavirus protein was stained with Cy3 anti-human IgG antibody. Nuclei were visualized by DAPI. Picture A shows the expression of hACE2 via the GFP tag. Picture B shows bound Fra1-S1-Fc. Picture C is the merge of all three stainings (S1-Fc, hACE2-GFP and nuclei)

In the same way, soluble forms of the spike proteins of two SARS-like bat coronaviruses, Bg08-S1-Fc and Rp3-S1-Fc, were analysed for binding to VeroE6 cells and HeLa cells transiently expressing hACE2. The assay revealed no background binding of Fc tag but also no binding of the bat-CoV-derived S1 spike fragments (figure 12).

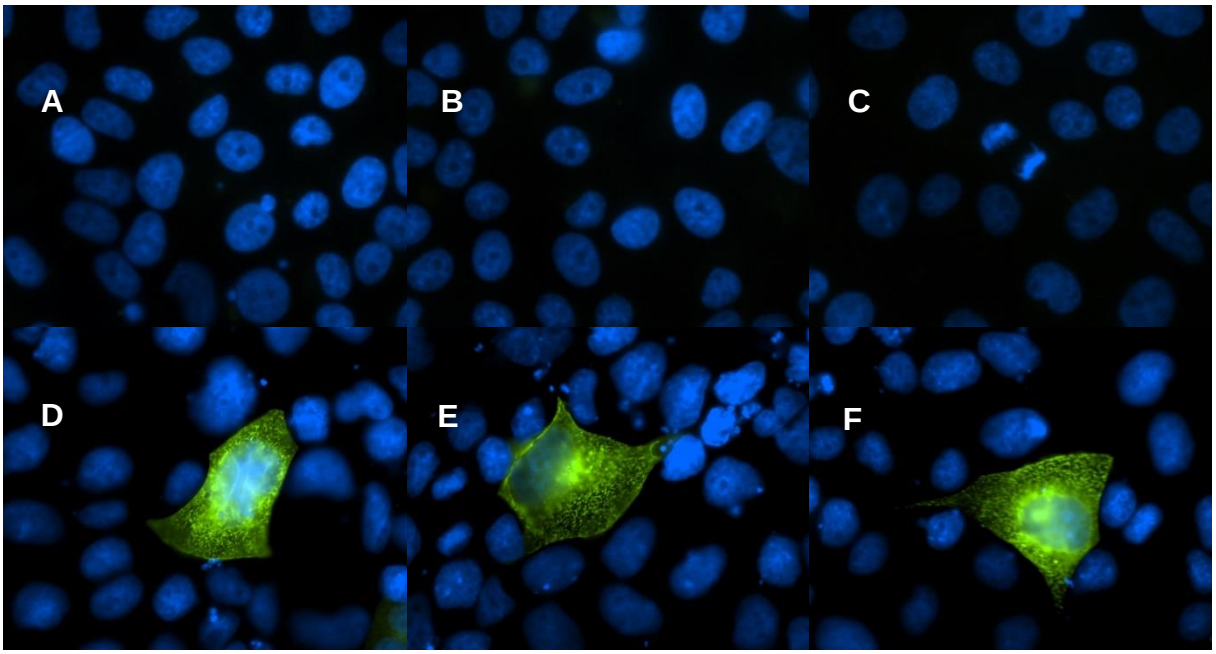

Figure 12: Binding of soluble bat-CoV spike protein to VeroE6 and cells expressing hACE2-GFP
Binding of soluble bat-CoV spike proteins to VeroE6 (A-C) or HeLa cells transiently expressing hACE2-GFP (D-F). Cells were stained for the detection of bound Bg08-S1-Fc (A,D), Rp3-S1-Fc (B,E) or ATG-Fc (C,F) with Cy3 anti-human IgG antibody. Cells expressing hACE2-GFP are detectable by the green fluorescence of GFP (D-E)

6.3 Binding of soluble spike proteins to chiropteran cells

In this study 26 chiropteran cell lines were used for the identification of a putative surface protein interacting with bat-CoV´s. All of these cell lines had been immortalized by the *Simian Virus* large T antigen. This set includes species from the two orders of *Micro*- and *Megachiroptera*. The cells were derived from different organs including the lung, kidney, brain, intestine, endometrium and uncategorized embryonic cells (table 4). To detect a specific interaction the soluble spike proteins were applied to these cells grown on coverslips and then stained by Alexa Fluor-488 anti-human IgG antibody. No binding was detected except for binding of Fra1-S1-Fc protein to VeroE6 cells.

Table 4: Chiropteran cells used for binding assay with soluble proteins
Displayed are all tested Chiropteran cell lines with their abbreviated name, the corresponding order and species from which the respective line was derived from and the donor organ.

	Species	**Cell line**	**Organ**
Primate	*Chlorocebus sp.*	VeroE6	
Microchiroptera	*Rhinolophus landerii*	RlKd	Kidney
	Rhinolophus alcyone	RhiLu-1.1	Lung
		RhiNi1.2	Kidney
		RhiBrain-4p	Brain
	Rhinolophus euryale	RhiEuLu	Lung
	Rhinolophus ferrumequinum	RhiFeLu	Lung
	Pipistrellus pipistrellus	PipNi-3	Kidney
		PipNi-4	Kidney
	Myotis daubentonii	MyDauDa-46	Intestine
		MyDauLu-47	Lung
		MyDauBrain-48	Brain
		MyDauBrain-48B	Brain
	Hipposideros caffer	HipEm-5	Embryonic
	Hipposideros caffer ruber	HipEm-28	Embryonic
	Hipposideros abae	HipaLu-24	Lung
		HipaLu-27	Lung
Megachiroptera	*Eidolon helvum*	EidNi-41	Kidney
		EidLu-43	Lung
	Rousettus aegyptiacus	RoEnd-4	Endometrium
		RoNi-7	Kidney
	Epomophorus	EpoNi-22.3	Kidney
	Hypsignathus monstrosus	HypLu-2	Lung
		HypLu-45	Lung
		HypNi-1	Kidney
		HypNi-21	Kidney
	Tadaria brasiliensis	Tb1Lu	Lung

6.4 Binding of soluble spike proteins to heterologous expressed human receptor candidates

For human coronaviruses, three cellular proteins have been identified as receptors for virus entry: hACE2, hAPN and hDPP4. To analyse whether bat-CoV spike proteins may interact with either of these receptors, hAPN and hDPP4 were included in the assay as described above. For both hAPN (figure 13) as well as hDPP4 (figure 14) no binding of either Fra-S1-Fc, Bg08-S1-Fc or Rp3-S1-Fc could be detected.

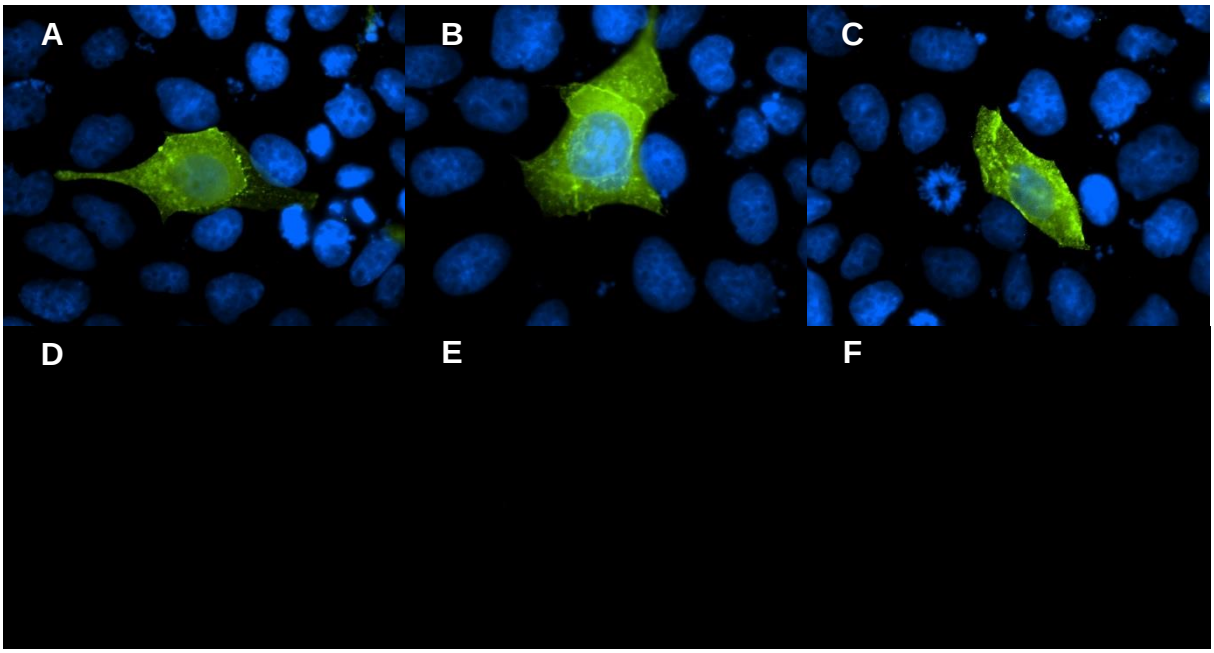

Figure 13: Binding of soluble spike proteins to hAPN-GFP
The pictures A-F show HeLa cells transfected for transient expression of hAPN-GFP. Pictures A-C reveal the expression of hAPN-GFP by the fluorescence of the GFP-tag, nuclei were stained by DAPI. The corresponding pictures D-F show the binding assay with Bg08-S1-Fc (A,D), Rp3-S1-Fc (B,E) and ATG-Fc (C-F) using Cy3 anti-human IgG antibody for staining bound Fc-tagged protein.

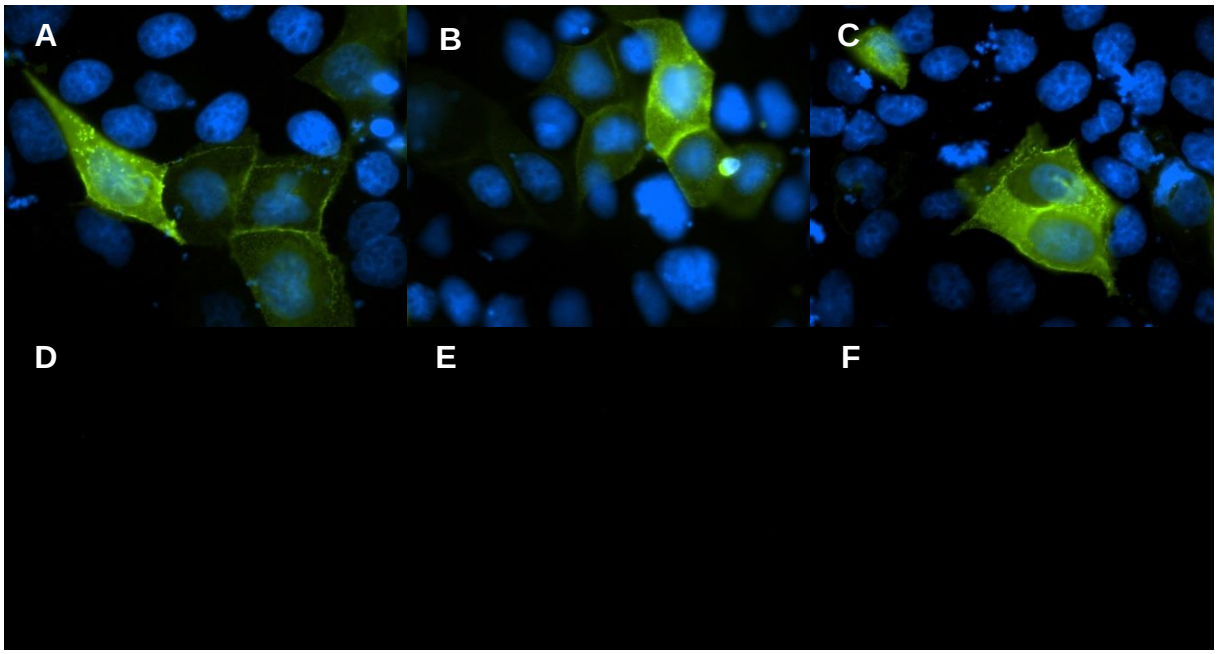

Figure 14: Binding of soluble spike proteins to hDPP4-GFP
The pictures A-F show HeLa cells transfected for transient expression of hDPP4-GFP. Pictures A-C reveal the expression of hDPP4-GFP by the fluorescence of the GFP-tag, nuclei were stained by DAPI. The corresponding pictures D-F show the binding assay with Bg08-S1-Fc (A,D), Rp3-S1-Fc (B,E) and ATG-Fc (C-F) using Cy3 anti-human IgG antibody for staining bound Fc-tagged protein.

6.5 Binding of soluble spike proteins to chiropteran receptor candidates

There are indications that bat coronaviruses may utilize receptors similar to their human counterparts. For that reason, it was attempted to isolate bat ACE2, APN and DPP4 cDNA. *Rhinolophus* cell lines were chosen as the bat-CoV spike proteins had been isolated from bats of the genus *Rhinolophus*.

Three different receptor candidates were successfully cloned. RL-ACE2 from a *Rhinolophus alcyone* lung cell line, RN-ACE2 from *Rhinolophus landerii* kidney cells as well as bDPP4 from *Rhinolophus euryale* lung cells. Rp-ACE2 from *Rhinolophus sinicus* was kindly provided Prof. Dr. Hongkui Deng. All four proteins were tagged with the green fluorescent protein to visualize the protein expression.

As the figures 14-17 show, no binding of the bat-CoV-derived soluble spike proteins was detected on cells expressing either of the four receptor candidates. However, Fra1-S1-Fc exhibits specific binding to cells expressing RN-ACE2-GFP.

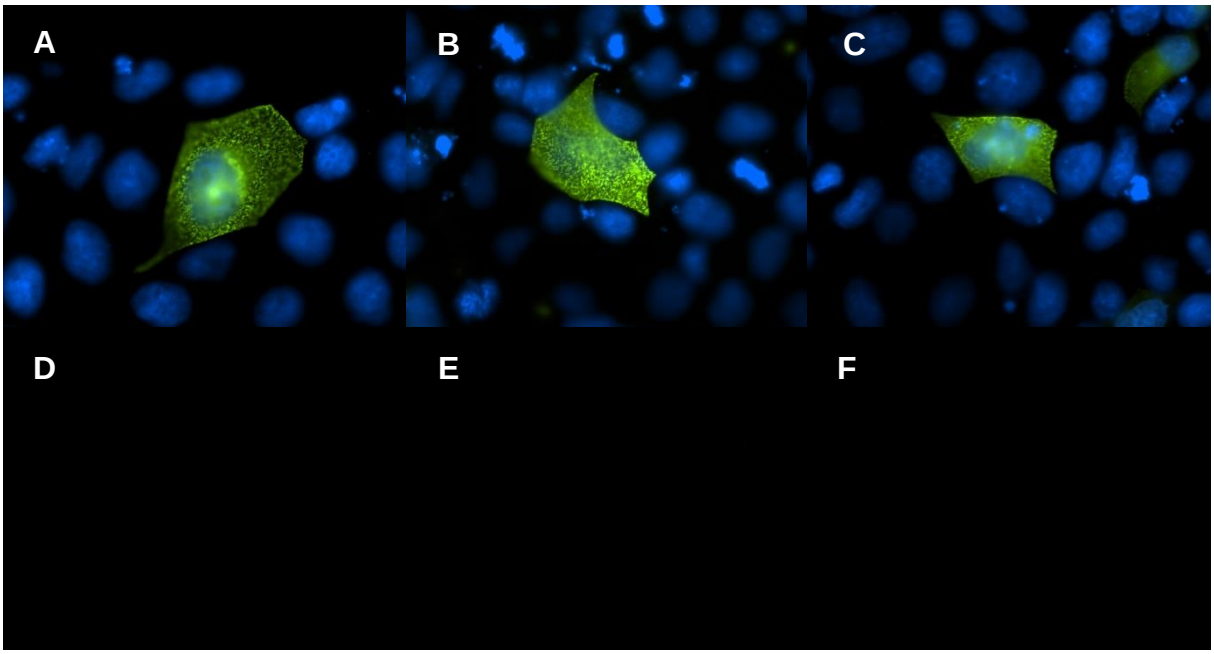

Figure 15: Binding of soluble spike proteins to RL-ACE2-GFP
The pictures A-F show HeLa cells transiently expressing RL-ACE2-GFP. Pictures A-C demonstrate the expression of RL-ACE2-GFP and the location of the DAPI-stained nuclei. Pictures D-F were stained with Cy3 anti-human IgG antibody for detection of bound Fra1-S1-Fc (D), Bg08-S1-Fc (E) and Rp3-S1-Fc (F).

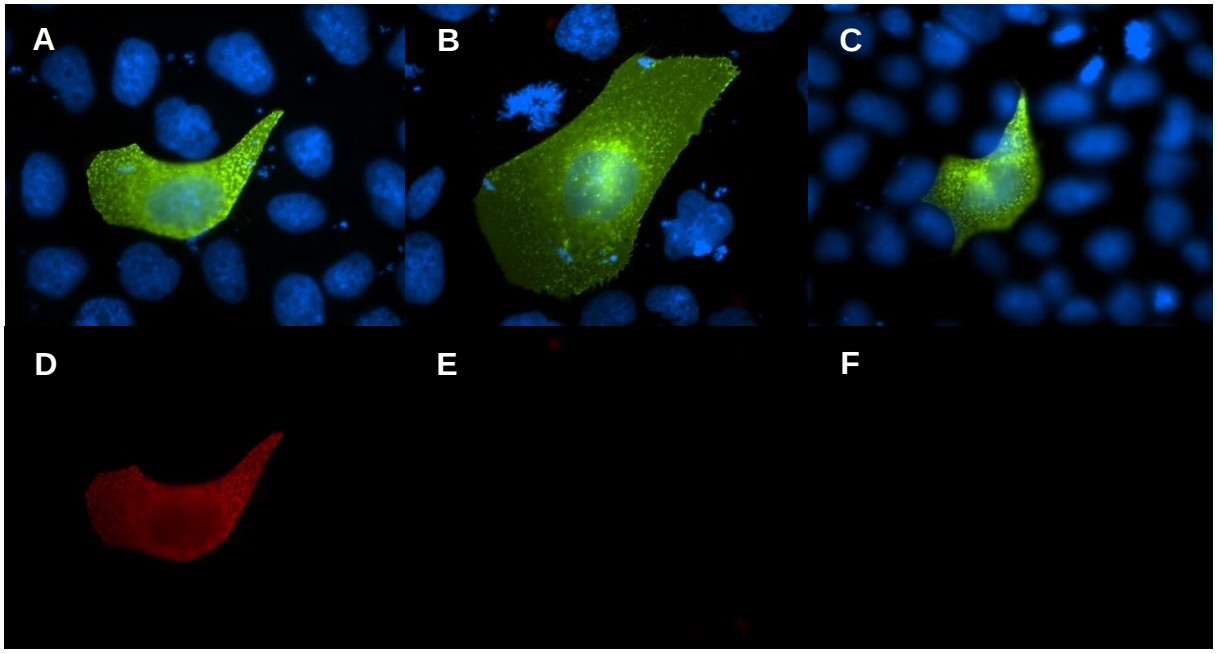

Figure 16: Binding of soluble spike proteins to RN-ACE2-GFP
The pictures A-F show HeLa cells transiently expressing RN-ACE2-GFP. Pictures A-C demonstrate the expression of RN-ACE2-GFP and the location of the DAPI-stained nuclei. Pictures D-F were stained with Cy3 anti-human IgG antibody for detection of bound Fra1-S1-Fc (D), Bg08-S1-Fc (E) and Rp3-S1-Fc (F).

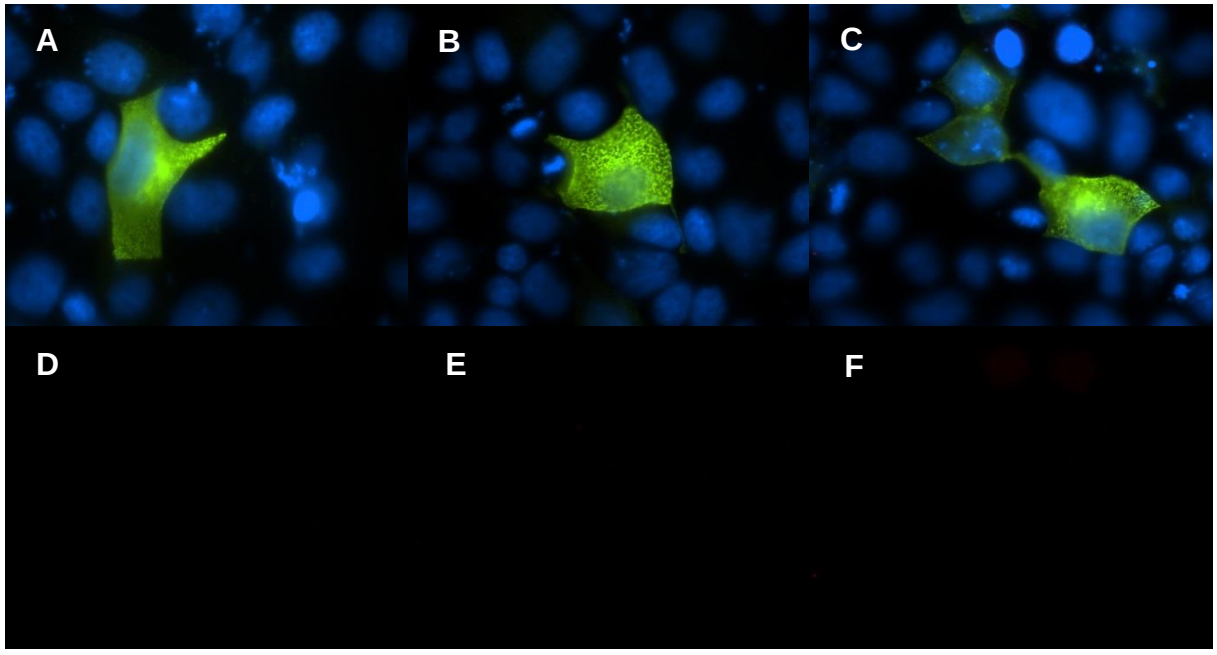

Figure 17: Binding of soluble spike proteins to RP-ACE2-GFP
The pictures A-F show HeLa cells transiently expressing Rp-ACE2-GFP. Pictures A-C demonstrate the expression of Rp-ACE2-GFP and the location of the DAPI-stained nuclei. Pictures D-F were stained with Cy3 anti-human IgG antibody for detection of bound Fra1-S1-Fc (D), Bg08-S1-Fc (E) and Rp3-S1-Fc (F).

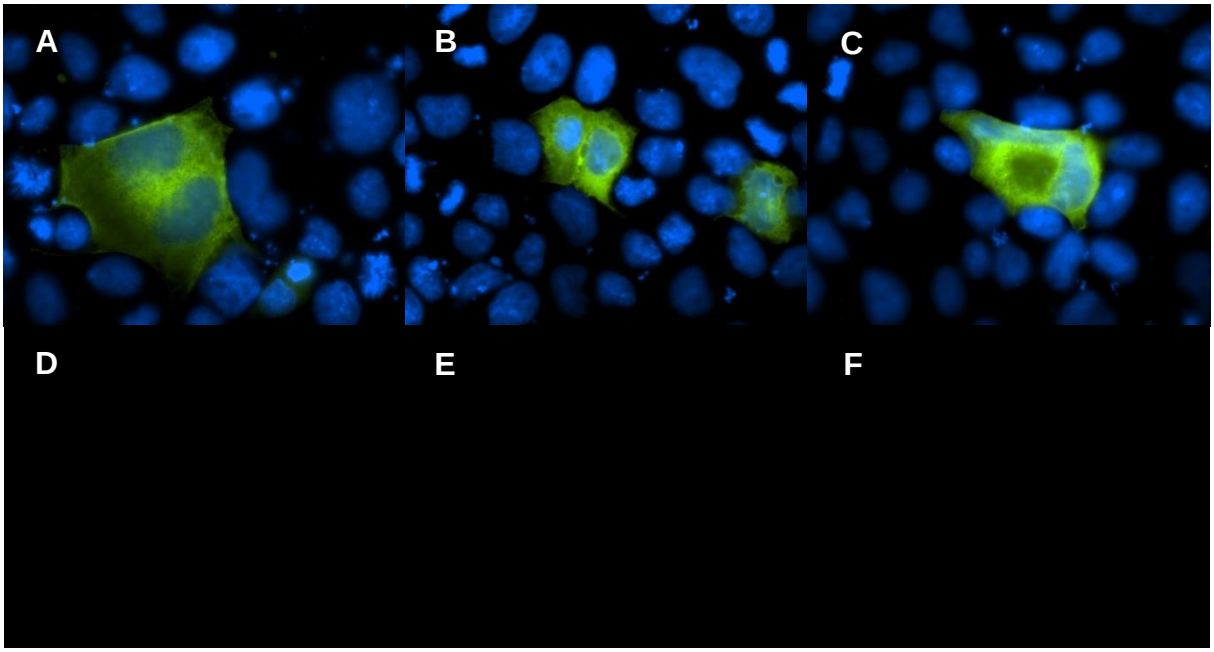

Figure 18: Binding of soluble spike proteins to bDPP4-GFP
The pictures A-F show HeLa cells transiently expressing bDPP4-GFP. Pictures A-C demonstrate the expression of bDPP4-GFP and the location of the DAPI-stained nuclei. Pictures D-F were stained with Cy3 anti-human IgG antibody for detection of bound Fra1-S1-Fc (D), Bg08-S1-Fc (E) and Rp3-S1-Fc (F).

6.6 Cell based binding assay with human or chiropteran cells

With the cell-based binding assay interaction between spike proteins and receptors can be analysed in a less artificial way compared to the soluble spike proteins. Whereas soluble spike proteins only contain the respective S1 subdomain and do not have the ability to form homo-trimers, here full-length spike proteins are expressed. These proteins have a cytosolic DsRed tag for identification by immunofluorescence. BHK-21 cells were transfected to express this proteins, detached from the culture dish and overlaid onto adherent cells of different origins. After stringent washing the bound BHK-21 cells where counted via their specific DsRed fluorescence and analysed whether there are significant differences between cells expressing one spike protein or another.

Initially it had to be shown that all three spike proteins analysed exhibit comparable expression and localization patterns. Transfected BHK-21 cells expressing DsRed show fluorescence distributed all over the cytoplasm which is also the case for the spike proteins (figure 19 A-D). There is no intracellular retention which has been reported for other coronavirus spike proteins. When transfected cell were grown on 10 cm dishes, both bat-CoV spike exhibit an overall lower DsRed intensity compared to Fra1-Sred or DsRed alone (figure 19 E-H), but the same transfection efficacy (figure 19 I-L).

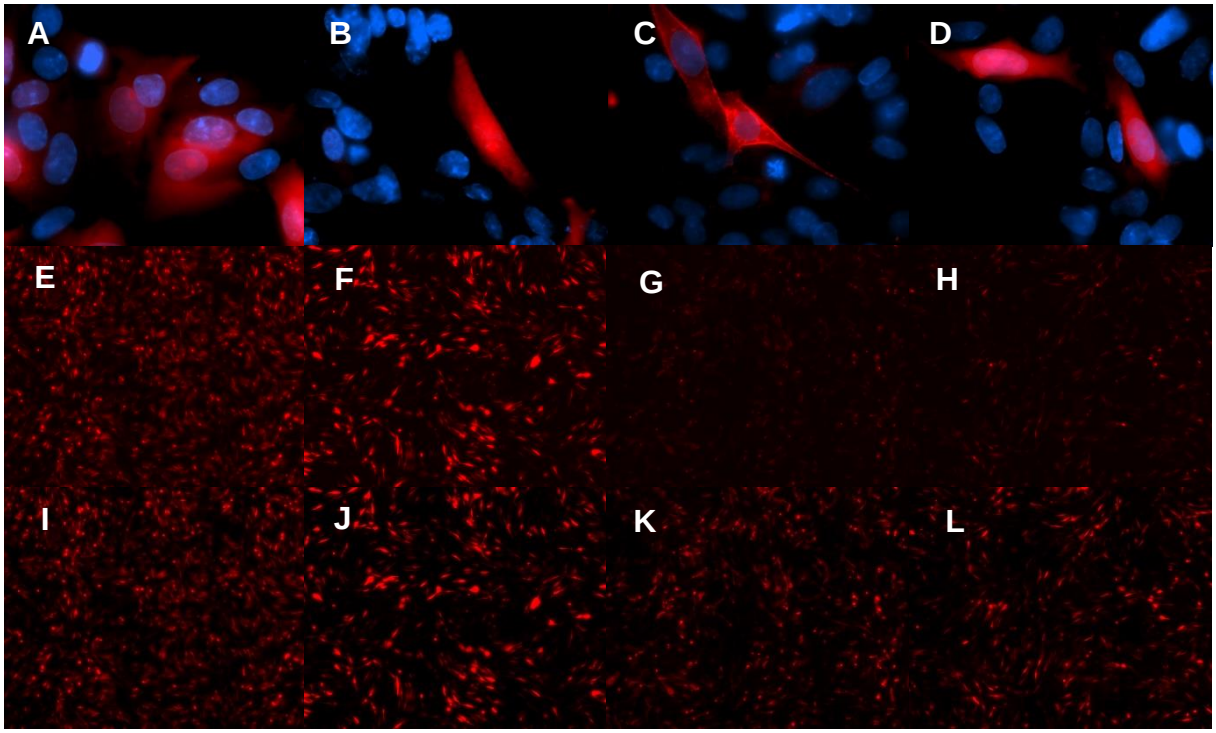

Figure 19: Expression of the DsRed tagged spike proteins in BHK-21 cells
This pictures show the expression of DsRed (A,E,I), Fra1-Sred (B,F,J), Bg08-Sred (C,G,K) and BB9904-Sred (D,H,L) in BHK-21 cells. The picture A-D were taken from coverslips samples at 100x -fold magnification, the pictures E-L from 10 cm dishes by 10x-fold magnification. The pictures E-H had the same exposition time whereas the pictures K-L the contrast and intensity was where matched to better visualize the overall amount of transfected cells, especially for Bg08-Sred and BB9904-Sred.

Figure 20 illustrates the differences that are observed when either transfected BHK-21 cells expressing DsRed or Fra1-Sred are applied to VeroE6 cells. These pictures are exemplary for the images which were the basis for the quantification. There were variations in the overall binding capacity of the tested primate and chiropteran cells which resulted in different numbers of bound cells. For this reason figure 21 shows the ratio of bound cells expressing spike protein in relation to bound cells expressing DsRed. The statistical analysis of the data revealed a significant increase in the amount of bound BHK-21 cells expressing Fra1-Sred ($p < 0.05\%$) to VeroE6 cells, but for none of the other samples analysed.

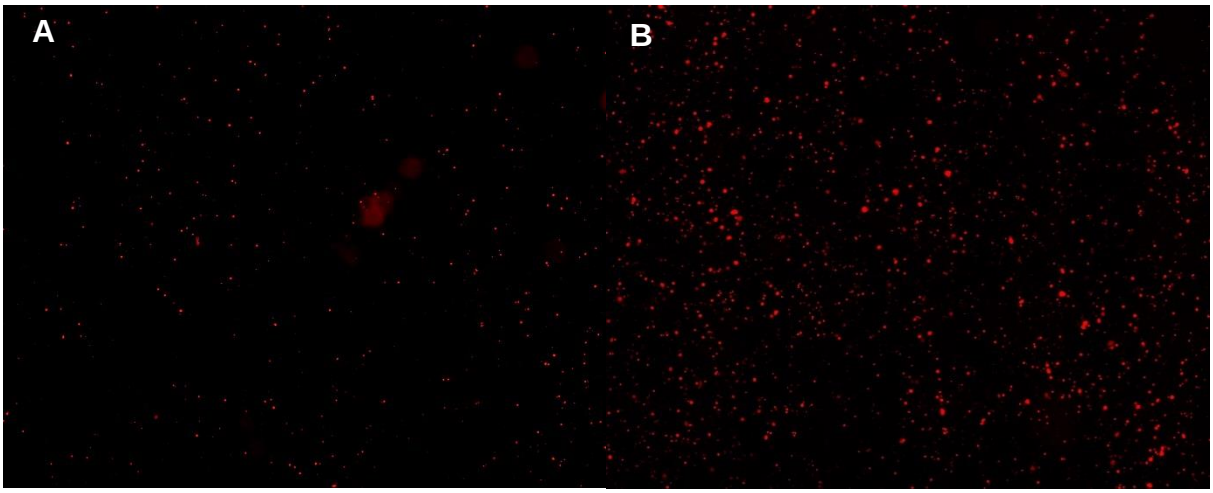

Figure 20: Cell based binding assay with VeroE6 cells
These pictures show BHK-21 cells either transfected with DsRed (A) or Fra1-Sred (B) bound to VeroE6 cells.

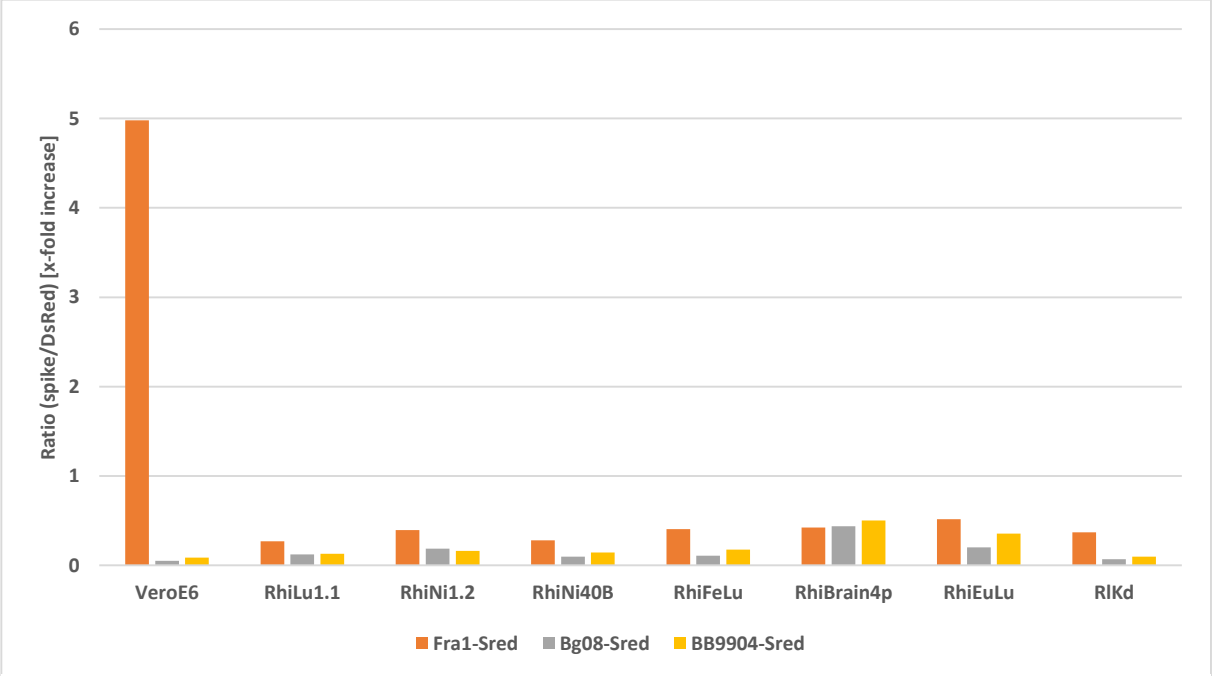

Figure 21: Cell based binding assay with *Rhinolophus* cells
Binding capabilities of BHK-21 cells expressing either of three different spike-DsRed proteins to VeroE6 cells and seven different *Rhinolophus* cell lines. It is indicated as the increase of bound BHK-21 cells expressing either of the spike-DsRed proteins compared to the negative control of DsRed expressing cells.

6.7 Cell based binding assay with heterologous expressed receptors

As there was no binding detectable between BHK-21 cells expressing bat-CoV spike proteins and any of the tested chiropteran cells, the next step was to analyse potential receptor candidates in an overexpression system. For this purpose, HeLa cells were transfected for expression of these candidate proteins and overlaid with BHK-21 cells expressing different spike proteins. Transfection of the HeLa cells with the various receptor candidates usually resulted in a coverage of about 8 % of the total surface area presenting receptor proteins (figure 22). The HeLa cells exhibit almost no binding capacity if not transfected with a receptor candidate (figure 23).

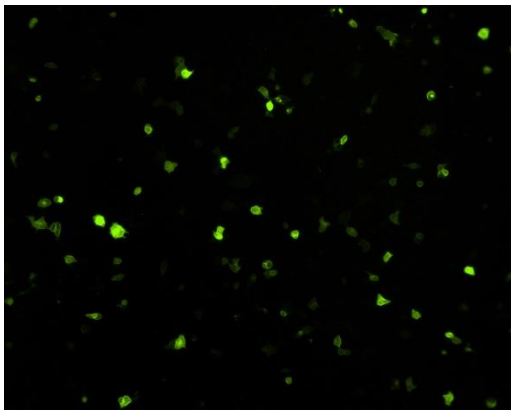

Figure 22: Transfection efficacy of HeLa cells
Transfections efficacy of HeLa cells transfected with hACE2-GFP at 10x fold magnification

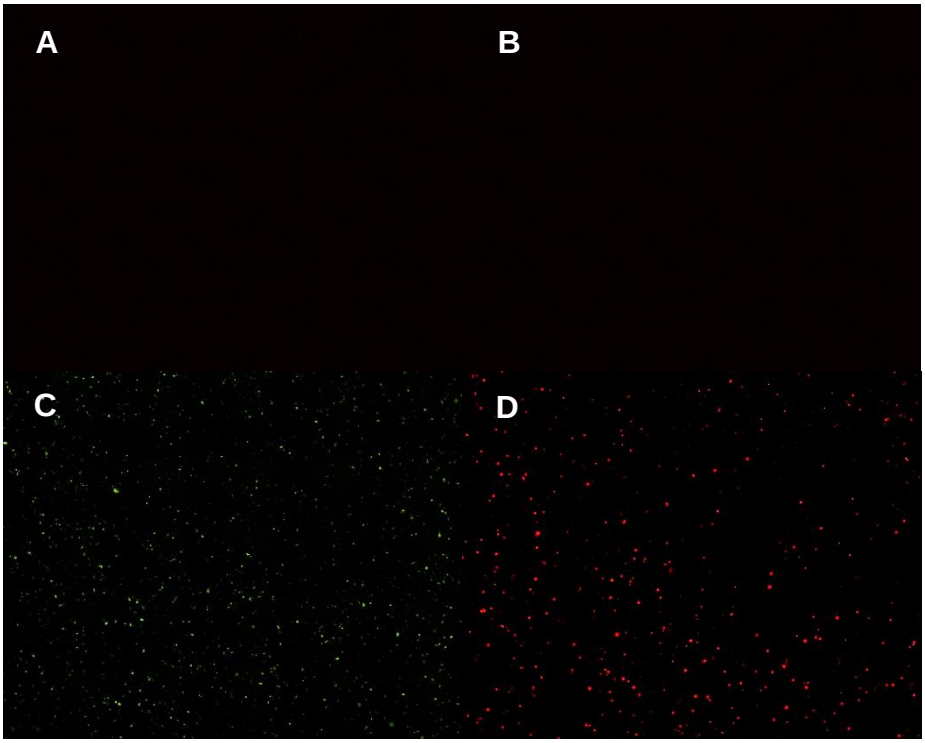

Figure 23: Binding capacity of transfected HeLa cells
The corresponding images of HeLa cells expressing no foreign protein (empty pCG1 vector) (A-B) or hACE2-GFP (C-D). Picture C shows the HeLa cells expressing hACE2-GFP whereas picture D shows the bound BHK-21 cells expressing Fra1-Sred.

Figure 24 shows the data obtained by comparing all seven potential receptor candidates as ratio of bound BHK-21 cells expressing spike proteins to cells expressing DsRed. The statistical analysis of the data revealed a significant ($p < 0.05\%$) increase in the amount of bound Fra1-Sred cells to cells expressing hACE2, RL-ACE2 or RN-ACE2. Furthermore, the differences between those three ACE2 proteins were also significant ($p < 0.05\%$) with hACE2 having the highest number of bound cells followed by RL-ACE2 and RN-ACE2. This result does not reflect the results of the assay with soluble spike proteins where only an interaction of the Fra1 protein with hACE2 or RN-ACE2 could be identified.

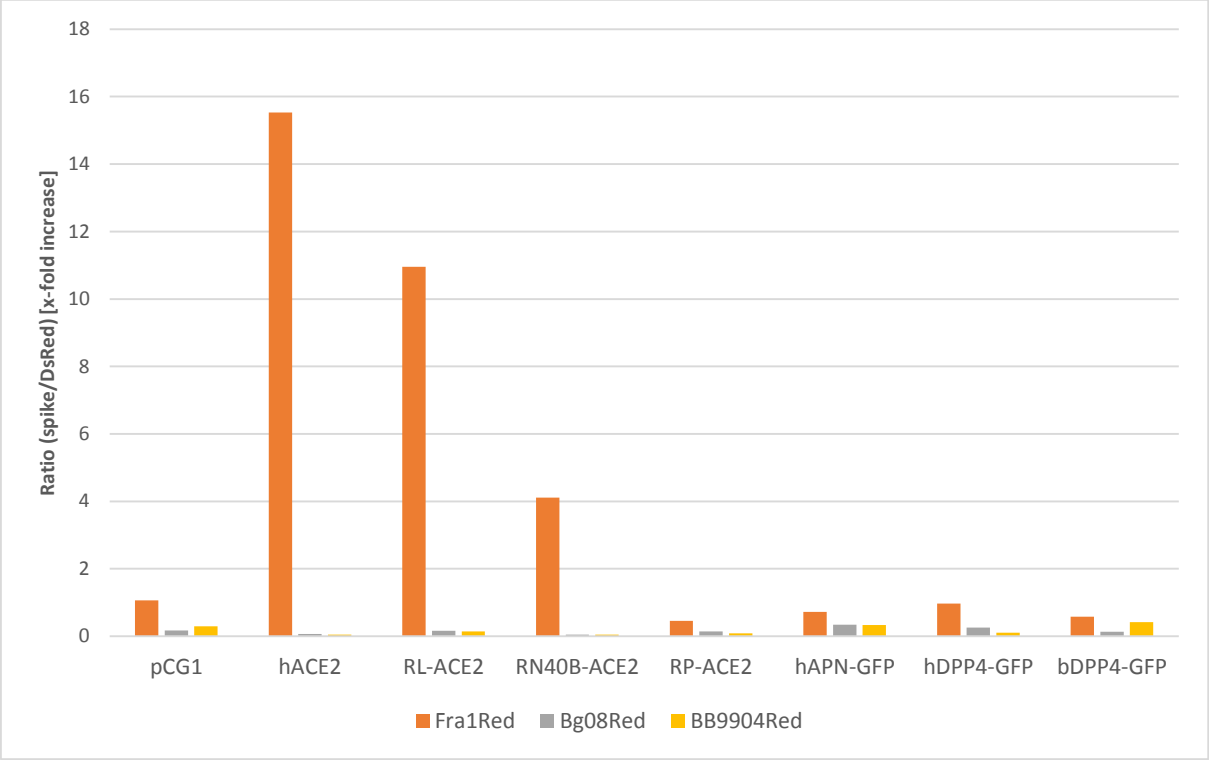

Figure 24: Cell based binding assay with transfected receptor candidates
Binding capabilities of BHK-21 cells expressing either of three different spike-DsRed proteins to HeLa cells expressing different receptor candidates. Shown is the ratio of bound BHK-21 cells expressing spike-DsRed transfected to cells expressing only DsRed.

6.8 VSV-pseudotype infection of cells expressing human or chiropteran receptors candidates

In this assay pseudotyped virions of *Vesicular Stomatitis Virus* (VSV) were used. The genome of this recombinant virus features a gene encoding firefly luciferase in place of the gene for the VSV G protein. This protein can be substituted by another viral surface protein if it is provided *in trans* by the cell infected by VSV-ΔG-G. It is known that coronavirus spike proteins can be functionally incorporated into VSV virions using this method. Infectivity of the pseudotyped particles can be quantified by the enzyme activity of the luciferase expressed in infected cells.

Two points were addressed with this assay. First, to control whether the failure of the bat-CoV spike proteins to bind in the assays describe above is a result of an overall weak affinity between spike protein and receptor. This affinity, too weak for the binding assays, might be sufficient to mediate infection of VSV-pseudotypes. Second, to evaluate whether the different binding of the Fra1 spike protein to the three different ACE2 proteins observed in the cell based binding assay can be shown also in an infectivity assay.

The results show a significantly ($p < 0.05\%$) increased infectivity of VSV-Fra1-S pseudotypes if one of the ACE2 (h/RL/RN) receptor where transfected. Also the infectivity was significantly higher if hACE2 serves as receptor as compared to both *Rhinolophus* ACE2´s. No significant difference was detectable between the two *Rhinolophus* receptors. None of the tested bat-CoV spike proteins mediated any significant infection.

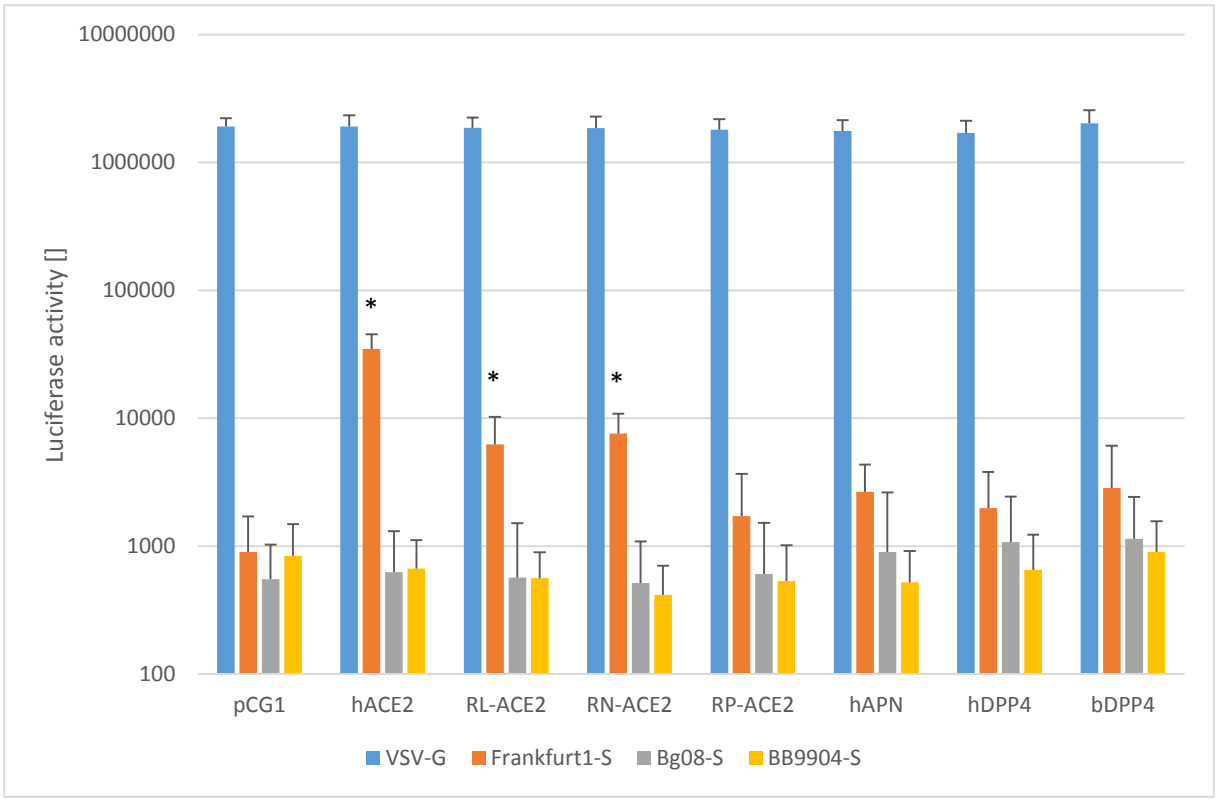

Figure 25: Luciferase Assay in total values
Luciferase activity after infection of BHK-21 cells transfected with different receptor candidates by VSV-pseudotypes with different fusion proteins. The asterisk indicate statistical significant increased values compared to the infection of cells transfected with an empty pCG1 vector.

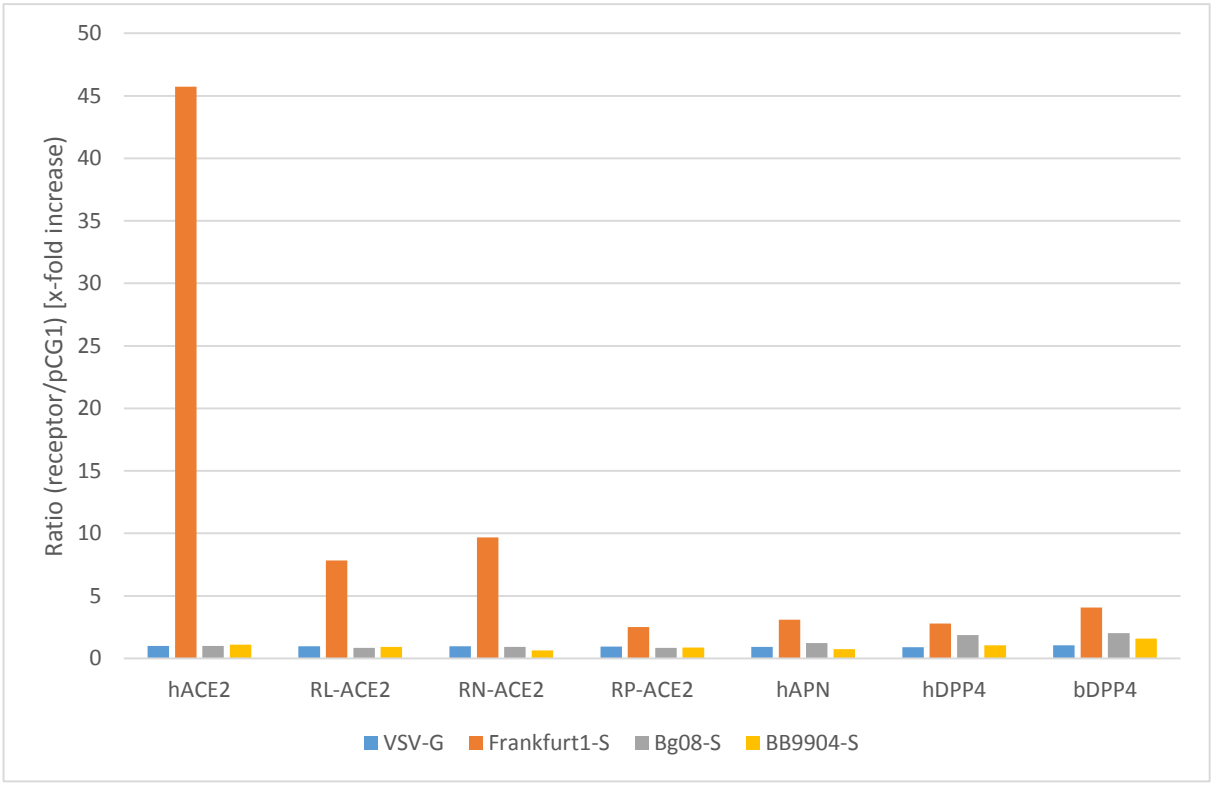

Figure 26: Luciferase Assay in relative values
Same data as in figure 25, but as a ratio of the values obtained by infecting those cells transfected with a receptor candidate to the value obtained by infecting pCG1 transfected cells with the same pseudotypes.

7 Discussion

Coronaviruses are a new emerging threat to public health. Formerly known as pathogens of mild respiratory infections in humans they were not in the focus of virology research. This notion changed with the SARS pandemic in 2002, causing over 800 fatalities and having a huge impact on the Asian economy, inflicted by the travel restriction and quarantine measures. It was even reinforced by the recent appearance of MERS-CoV in 2012, which displays an equally high pathogenicity as SARS-CoV.
The emergence of SARS-CoV demonstrated how vulnerable modern society has become by means of intercontinental travel. After reaching the Hong Kong area with its possibilities of transportation, SARS-CoV rapidly spread into over 30 different countries worldwide. But it also showed how capable the public health sector and medicine in general have become, as even after the distribution to so many places worldwide, the imposed quarantine measures ended this pandemic efficiently.
The community of virologists showcased their prowess as they rapidly identified the pathogen, its complete genome sequence and even the cellular receptor in just a couple of months.

7.1 SARS coronavirus as an exemplary zoonosis

Zoonotic pathogens are the dominant cause of emerging infectious diseases in humans and RNA viruses build the majority among them[74, 147]. The nature of their RNA genome with relative high mutation rates probably helps these pathogens to evolve more rapidly and adapt faster to new hosts, as for example bacteria or even DNA viruses could. This feature is one of the difficulties researcher face when they try to uncover the origins of such pathogens.
The emergence of SARS was most likely one of the best documented zoonotic events in human history. During all stages of this pandemic samples have been collected and virus sequenced. It offered insights into the adaptation process this virus underwent while spreading. Additionally, entire genomes of probable precursor viruses from a suspected intermediate host[60] as well as from their bat reservoir are now available[87]. Combined with the knowledge obtained by studies of different other coronaviruses we have a more detailed view of the emergence of this RNA virus than we probably ever

had for any other. Especially the thorough analysis of the fusion protein receptor interaction by co-crystallisation and the progress in reverse genetics helps to unravel the molecular biology of this virus in ways unimaginable of few decades ago.
Benefiting from this advance, we directed our studies to help understand what obstacles had to been overcome to get this virus transmitted from bats to humans. Therefore the identification of the receptor for bat SARS-like CoV was the major aim of this project.

7.2 Human ACE2 is the functional receptor for SARS coronavirus

The identification of the viral receptor opened up an important topic in research of the genesis of SARS-CoV. Studies of different coronaviruses like IBV, MHV and FCoV established the notion that receptor recognition is one if not the major barrier for coronaviruses to be transmitted between species. It had been proven before that just small changes to the amino acid sequence of coronavirus spike proteins can alter the tissue tropism and make this viruses even transmissible to new hosts[18, 62, 81, 131].
Based on these findings interests focused on the efficiency of receptor recognition by SARS-CoV. Studies analysed to what extent the spike proteins of SARS-CoVs from different stages of the pandemic can utilise human ACE2. Interestingly, the efficiency of binding ACE2 decreased between spike samples form the early stages during 2002/3 compared to some taken from a small outbreak 2003/4 with less severity and no reported human-to-human transmission. The comparison to civet ACE2 and SARS-like CoV from civets was even more surprising as the spike proteins from the civet SARS-like CoV and from the 2003/4 virus both bound less efficient than this from 2002/3[94]. These results also correlated to the observation that the sampled civet cats showed no clinical symptoms[60] but diseased when challenged with SARS-CoV from 2002/3[174]. In sum these finding suggest that the extent and severity of infection could correlate to the efficiency of ACE2 binding.
A successful crystallisation of the soluble spike RBD in complex with human ACE2 later uncovered the complete nature of the binding between fusion protein and receptor and resolved the interacting regions as well as all responsible amino acids[91].
In 2004 another human alphacoronavirus, HCoV-Nl63, was isolated and subsequent studies showed that this virus despite being only distantly related to SARS-CoV employs the same receptor and even the sites of binding are overlapping[67, 95].

Crystallisation studies later revealed that the spike RBDs of both viruses lack any significant structural homology[175].

7.3 Bat ACE2 as a functional receptor for SARS coronavirus

The results showing that SARS-CoV and the SARS-like CoV from civets utilises ACE2 as receptor and the studies of HCoV-NL63 in addition, raised the question what receptor the precursor of SARS-CoV use. Several SARS-like CoV have been identified in *Rhinolophus* species in the region of Hong Kong and south-east China. They share an identical genome organisation and very high sequence identity to SARS-CoV, with exception of the spike S1 domain and ORF 8. The closest known bat SARS-like coronavirus (Rp3) was isolated from a *Rhinolophus pearsonii* bat in south-east China and features a 92% amino acid identity to SARS-CoV overall, but only 64% at the S1 domain including two larger deletions[80, 93, 122]. Researcher conducted experiments to compare SARS, civet and Rp3 CoV spike proteins in their usage of ACE2 as a receptor. It revealed that *R.pearsonii* ACE2 was not utilised by any of these three spike proteins and the Rp3 spike could also not recognize human or civet ACE2[123]. These findings let to the notion that the precursor of SARS CoV did not utilise ACE2 and strongly suggested that a switch in receptor usage was part of the zoonotic event. Further evidence supporting this hypothesis was found by another research group which managed to construct a complete cDNA clone of a bat SARS-like CoV, but were unable to rescue live virus on cells permissive for SARS-CoV. Only by exchanging the entire S1 subdomain with that from SARS-CoV virus could be rescued, proving that efficient recognition of human ACE2 is sufficient for bat SARS-like CoV to infect human and murine cells *in vitro*[6].

However, in 2010 Hou *et al.* reported successful infection of cells expressing different bat ACE2s by pseudotypes with the SARS-CoV spike protein. They had tested ACE2s of five different *Rhinolophus* species as well as *Hipposideros pratti* and a *Myotis daubentonii*, all resident in south-east China. From all nine proteins only the *Rhinolophus sinicus* and the *Myotis daubentonii* ACE2 mediated permissiveness in their experiments[71]. In the same year also the ACE2 of *Rousettus leschenaultii* was tested positive[184].

The data presented in this thesis confirm that the SARS-CoV spike protein is able to utilize ACE2 of two additional *Rhinolophus* species as a functional receptor. In total there are now 5 different bat species known to support SARS-CoV infection in regard to receptor specificity. So there are proper receptors available in bat species and a switch in receptor specificity was not inevitable for the SARS-CoV precursor virus to cross the species barrier.

7.4 Precursor of SARS coronavirus utilise bat ACE2 as a receptor

Coronaviruses exhibit a very dynamic genome composition with strong indication for frequent recombination events in nature. There is also abundant evidence for a multitude of successful cross species transmission. As we now know that SARS-CoV can utilise civet as well as several bat ACE2s as receptor, the question remains if this receptor specificity was already present in bat SARS-Like CoV or has developed in civet cats serving as an intermediate host. I think there are some points supporting the first hypothesis.

To begin with is the ability of HCoV-NL63 to utilize human ACE2. The branch between alpha- and betacoronaviruses has to have happened a very long time ago and both genera have acquired distinct genomic features. Similarities of the spike S1 domain between HCoV-NL63 and SARS-CoV are almost non-existent, but despite that both viruses utilize the same host receptor and actually at almost the same epitope[95]. There are two possible courses of events. One or both viruses just recently acquired the ability to utilise ACE2 as receptor, during or after their transmission to humans. Or, both CoVs had spike proteins already selected to recognise protein structures very similar to human ACE2, which would be most likely bat ACE2. The latter hypothesis would include, that the ability to use ACE2 must have been present in bat coronaviruses for such a long time, that the structural differences in the S1 domains could have accumulated to form so different RBD structures. This force to diversify could be explained by the fact that spike proteins as the major viral antigen needs constant modification to escape the host immune response, if the virus has to persist in one population. In this regard it is interesting that ACE2 protein of known bat species features a remarkable high variation when compared to other mammals[71]. The combination of both facts fits a proposed model about the arms race between fast evolving pathogens and slowly evolving host, that predicts such diversity on the host

side to counteract the faster virus adaptability and thereby efficient receptor recognition[103]. Just recently a study combined the data on the crystal structure the SARS CoV spike complexed with human ACE2 and the available sequences of ACE2s. With the exact knowledge of interacting amino acids during receptor binding, they looked for processes of positive selection in *Rhinolophus* ACE2s. Their analysis revealed a strong statistical evidence for positive selection on 19 amino acids, 17 of which are localized in the region of binding and 6 at positions where human ACE2 directly interacts with the SARS CoV spike. They came to the conclusion that this is convincing evidence for the existence of bat coronaviruses utilising this receptor and a long lasting intimate co-evolution[36]. In my opinion this notion is further supported by the findings of Hou *et al.* as they not only identified two different alleles of the ACE2 protein present in *R.sinicus*, but could also demonstrate that just one of them is supporting SARS-CoV infection. This nicely fits the assumption that bats evolve ACE2 variants to counteract a constant challenge of coronavirus infection.

On a closer look at the crucial amino acids in all of the so far tested ACE2s more interesting details appear. In table 5 (supplement) we see that the *Rhinolophus* ACE2s share an overall higher homology to the human protein as the civet ACE2 does, specifically looking at the amino acids important in receptor binding. The ACE2 of *R.leschenaulti*, *R.pearsonii* or *R.sinicus* appear to be better suited to support a coronavirus which spike protein is also able to utilise human ACE2. If we just consider this point it seems more plausible that the precursor of SARS-CoV has its reservoir in one of these species than rather in palm civet cats. To further elaborate this point it would be interesting to see how the civet SARS-CoV spike protein interacts with the different bat ACE2s. If this spike protein is unable to utilise them it would be another evidence that palm civets were not an intermediate host.

I freely admit that the stated indications are mostly hypothetical and fail to explain the major contradiction to this perspective, which is the lack of the Rp3 spike protein to recognise any ACE2 protein. Despite being so closely related to SARS-CoV this virus appears to utilise a completely different receptor. One reasonable explanation would be that the low sequence identity in the S1 domain can be accounted to a recombination event and indeed has a phylogenetic analysis of the Rp3 genome revealed significant discordance to the SARS-CoV genome, indicating that Rp3 is a result of recombination with bat CoV lineage even closer related to SARS-CoV[69].

7.5 Bat betacoronaviruses utilise an unknown receptor

This project aimed to identify the natural receptor of bat betacoronaviruses. Our assays covered binding to proteins presented at the plasma membrane, as well as the possible interaction in endosomal compartments by the VSV-pseudotype assay. Many different bat species and cell types were tested but no positive results could be obtained.

Like the data of the parallel tested SARS-CoV spike protein clearly shows, do the applied assays miss the dominant pool of proteins which are not constitutively produced. By artificial overexpression of bat ACE2 and bDPP4 as well as hACE2, hDPP4 and hAPN, we conclusively demonstrated that these known coronavirus receptors are not utilised. Our attempts to isolate *Rhinolophus euryale* APN were unsuccessful, but considered that many alpha- and betacoronaviruses are known to use this receptor, it still is a valid candidate and should be evaluated in future studies.

Some coronaviruses do not even depend on a specific protein receptor und utilise sialic acids as attachment structures, especially many betacoronaviruses[133, 134, 136, 162, 171].

An excellent way to assess these receptor candidates is by hemagglutination of erythrocytes. We could therefore use our VSV pseudotypes or cells transiently expressing the bat CoV spike proteins, to test if these proteins possess hemagglutinating activity.

However, in my opinion the most plausible explanation for the negative outcome of our assays must be the insufficient expression of the receptor in our immortalised bat cell lines. The possible reasons for that are plenty, but it could be related to the transformation process, which most likely led to a dedifferentiation of the cells, or maybe settings like a missing hormonal stimulation.

7.6 Comparing binding and infection assays

In this project three different assay systems have been used, two of them based on the binding capacity of the spike proteins, whereas the pseudotype assay evaluated the functional utilisation of the receptor.

While binding tests with soluble spike proteins appears to be a convenient method to screen for interaction partners, this assay has one major limitation, which are the extensive modifications to the protein structure that are necessary to obtain soluble constructs. Despite the fact that we achieved to yield dimerised S1 proteins it is still

just an approximation to the full length homotrimer, which offers moreover trivalent binding of the receptor. Apparently, the binding capacity of soluble Fra1-S1-Fc does not suffice to reliably detect RL-ACE2-GFP, while the our other assays could demonstrate such interaction. Other publications reported the production of soluble S1 trimers utilising the GCN4 leucine zipper motif[13], but this method also seems to influence the natural binding activity[139].

Nonetheless are the soluble proteins a valuable tool because their application is not limited to this kind of binding assays. If we had found a positive cell line expressing a suitable receptor, the soluble spike proteins would have been applied in the identification of this protein. The Fc-tagged proteins for example could have been immobilised on protein A sepharose and then exposed to whole cell lysates or fractionised cell surface proteins. In this way a receptor protein could have been trapped and subsequently identified by protein sequencing. Another way would have been the separation of surface proteins of a permissive cell line via 2-dimensional SDS-PAGE, followed by western blot. Receptor candidates could thereby have been detected by binding of the soluble spike proteins to specific spots on the membrane, given that the receptor epitopes are mostly linear and still recognisable after the SDS treatment.

The cell based binding assay offers the quantitative analysis that the binding of soluble spike proteins cannot provide. It furthermore avoids the extensive amino acid modifications necessary for the construction of soluble spike proteins, besides the DsRed tag. However, as there are no commercial antibodies available that recognise the bat CoV spike proteins, a protein tag is necessary either way to ensure proper expression and localisation of these proteins. While the binding of the soluble proteins only indicated the binding of Fra1-S to the *Rhinolophus* ACE2s, the cell based binding assay not only confirmed this result but further revealed a significant difference in the binding capacity when compared to hACE2. Of course, this assay has its limitations and the results can only indicate distinct affinities of Fra1-S to the receptors. For example, differences in the amount of expressed ACE2 proteins or in their transport to the cell surface could influence the outcome. This could be addressed by quantifying the amount of GFP in whole cell lysate and biotinylated surface proteins via western blot analysis. However, to conclusively characterise this protein-protein interaction assays like the surface plasmon resonance analysis would be the ideal way to proceed.

Obviously the VSV pseudotype assay has the same inherent constraints concerning the comparable expression of the different receptor candidates. It also had to be demonstrated that all of the spike proteins are efficiently incorporated into VSV particles. As we did not had a specific antibody detecting all spike proteins and C-terminal modifications strongly interfere with incorporation, we had to address this problem by the construction of chimeric spike proteins. For this purpose my colleague Markus Hoffmann used the Fra1 and Bg08 spike protein with interchanged S1 domains. In a VSV pseudotype assay he could show that virus particles with spike proteins, consisting of a Fra1 S1 domain on a Bg08 backbone, could infect almost as efficient as VSV pseudotypes with the original Fra1 spike protein. Therewith was shown that bat CoV spike proteins are equally efficient incorporated into VSV particles as the Fra1 spike protein. In confirmation of the results I obtained with the soluble spike proteins, he also tested all cell lines of the 14 different bat species. He tried to infect them with VSV pseudotypes carrying either original Fra1 and Bg08 spike proteins or their chimeric variants, but found none of the cell lines to be susceptible. In this regard the pseudotype assay only confirmed the results already obtained through the bindings assays. Still, attachment is only first small foothold and functional utilisation of a receptor candidate can only be proven by infection.

7.7 Outlook

The exact nature of the interaction between the SARS-CoV spike protein and the ACE2s of human, civets and bats is still an interesting topic to cover. Also the question whether civets served as an intermediate host during the emergence of SARS-CoV cannot be satisfyingly answered, without the precursor virus. For this purpose, project partners are working on creating bat cell lines that overexpress those bat derived receptor candidates we isolated. This could help to finally accomplish a successful isolation of a bat betacoronavirus as well as basis for further research. With the identification of *R.alcyone* and *R.landerii* ACE2 as functional receptors for the SARS-CoV spike protein, these species should be included in future attempts to solate virus from free-living bats. But the phylogenetic analysis indicates that the search for the SARS-CoV precursor virus should not only be restricted to *Rhinolophus* bats as the ACE2s of other genera seem to be even closer related. Especially the local *Rousettus*

species in China appears to be another interesting candidate to look at. The experiments of Hou *et al.* also revealed the presence of multiple alleles of ACE2 in the same bat species. As a result, species known to act as a reservoir for SARS-like CoV should be analysed on their genetic variability concerning receptor candidates.

The identification of unknown virus receptors has been a major challenge ever since the beginnings of virus research. We have proven that the bat cell lines at our disposal are not susceptible to VSV pseudotypes with the bat SARS-like CoV spike proteins. Also binding of soluble or full length spike proteins could not be detected. This leads me to the conclusion that a proper interaction partner is insufficiently expressed in these cell lines. On this basis, we successfully isolated two potential receptor candidates of *R.alcyone*. These both proteins, ACE2 and DPP4, as well as human ACE2, APN and DPP4 have now conclusively proven to not act as a functional receptor for SARS-CoV. The remaining bat APN therefore is an interesting candidate, but we can also not eliminate the possibility that multiple alleles of receptor candidates exist and await their unravelling. Our group has also started the establishment of primary cell cultures in form of tissue slices, to screen bat lung and intestine for VSV-pseudotype susceptibility.

The most promising approach in my opinion would be the high-throughput screen of a cDNA library, which was already successfully used for numerous viruses before[9, 37, 119, 143]. Unfortunately, despite the amazing features of bats they have not been of much interest for the molecular biology and most of the tools, are simply not available for these animals. With the grown interest of infection biologist to them in recent years we will hopefully see this deficiency remedied.

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9 Supplement

9.1 Amino acids

One-Letter-Code	Three-Letter-Code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proliine
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
U	Sec	Selenocysteine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

9.2 Comparison of different ACE2 proteins

Table 5: Suceptible and non-susceptible ACE2 proteins

hACE2 pos.	24	27	31	34	37	38	41	42	45	79	82	83	90	325	329	330	353	354
H.sapiens	Q	T	K	H	E	D	Y	Q	L	L	M	Y	N	Q	E	N	K	G
P.larvata	L	T	T	Y	Q	E	Y	Q	V	L	T	Y	D	Q	E	N	K	G
R.alcyone	L	I	N	S	E	N	H	Q	L	H	N	F	N	E	N	N	K	G
R.landeri	L	T	D	S	E	N	Y	Q	L	H	N	F	N	E	N	N	K	G
R.sinicus Hubei	R	T	E	S	E	N	Y	Q	L	L	N	Y	N	E	N	N	K	G
R.leschenaulti	L	T	K	T	E	D	Y	Q	L	L	T	Y	D	E	E	K	K	G
M.daubentonii	K	I	N	S	E	D	H	E	L	L	T	Y	N	P	N	N	K	G
R.pearsonii muta	R	T	K	H	E	D	Y	Q	L	L	D	Y	N	E	N	N	K	D
R.pearsonii	R	T	K	H	E	D	H	E	L	L	D	Y	N	E	N	N	K	D
H.pratti	L	E	K	T	E	D	H	L	L	R	D	Y	N	K	E	N	K	G
R.macrotis	E	K	K	S	E	D	Y	E	L	L	N	Y	N	E	N	K	K	G
R.ferrumequinum	L	K	D	S	E	N	H	Q	L	L	N	F	N	E	N	N	K	G
R.pusillus	K	K	D	S	E	D	Y	Q	L	I	N	Y	T	E	N	N	K	G
R.sinicus Guangxi	R	I	K	T	E	D	H	Q	L	L	N	Y	N	E	N	N	K	G
M.musculus	N	T	N	Q	E	D	Y	Q	L	T	S	F	T	Q	A	N	H	G
R.rattus	K	S	K	Q	E	D	Y	Q	L	I	N	F	N	P	T	N	H	G
SARS	N473	Y475	Y475 Y442	Y440 N479	Y491	Y436	Y484 T486 T487	Y436 Y484	Y484	L472	L472	N473 Y475	T402	R426	R426	T486	G488 T487 Y491	Y491 G488

* The upper box lists all ACE2 proteins which act as receptor for SARS-CoV, the lower box all who do not. The box at the lower end shows the amino acids of the SARS-CoV spike protein which directly interact with human ACE2. Green areas show amino acids identical to human ACE2. Yellow areas show amino acids which exist in civet but not in the human ACE2. R.pearsoni muta = modified ACE2 aa40-42 SHE -> FYQ ;R.sinicus HB = Hubei province; R.sinicus GU = Guangxi province

9.3 Phylogenetic tree of ACE2 proteins

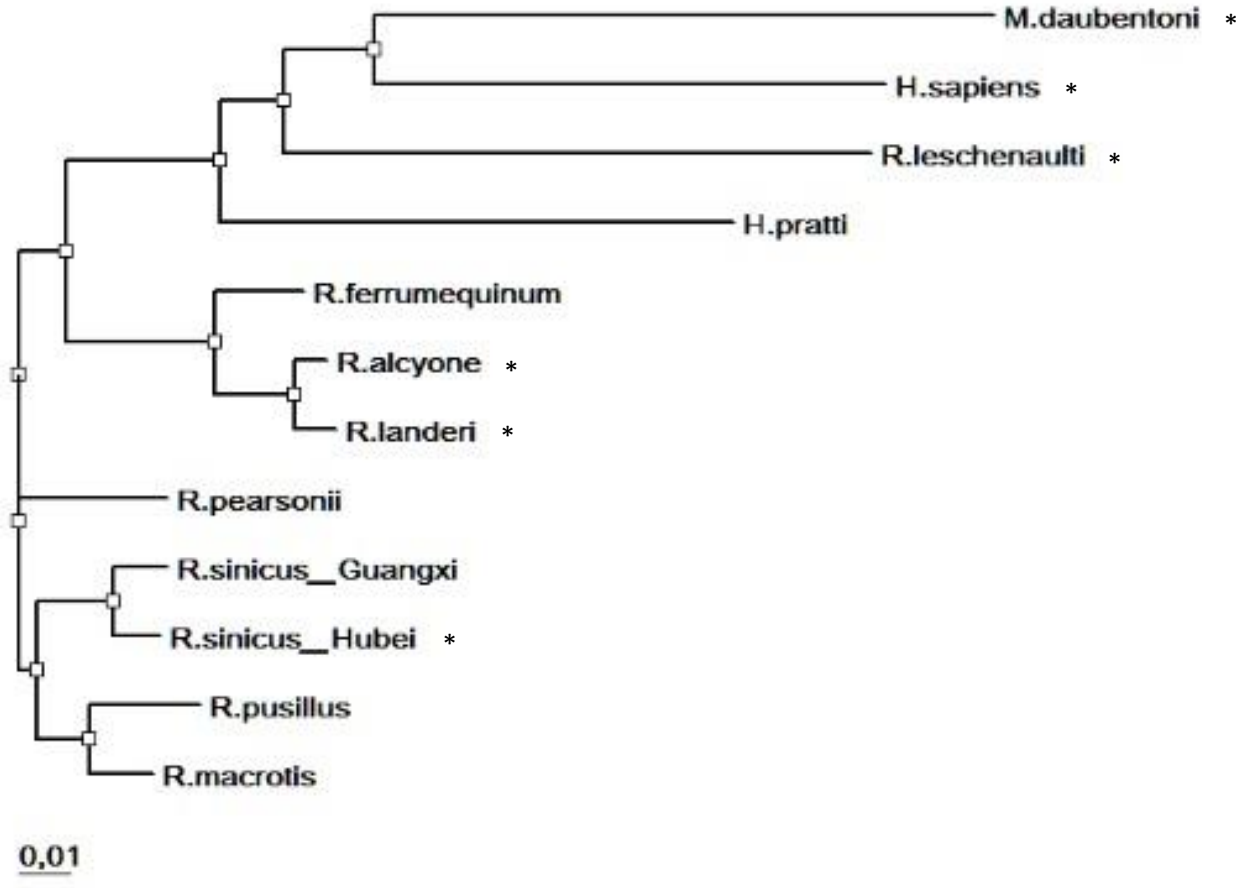

Figure 27: Phylogenetic tree of different ACE2 amino acid sequences
Based on full partial and full length amino acid sequences, created by Bayesian Alignment. ACE2 supporting SARS-CoV infection marked by asterisk.

9.4 Sequences

>Fra1-Sred

ATGTTTATTTTCTTATTATTTCTTACTCTCACTAGTGGTAGTGACCTTGACCGGTGCACCACTTTTGATGATGTTC
AAGCTCCTAATTACACTCAACATACTTCATCTATGAGGGGGGTTTACTATCCTGATGAAATTTTTAGATCAGACA
CTCTTTATTTAACTCAGGATTTATTTCTTCCATTTTATTCTAATGTTACAGGGTTTCATACTATTAATCATACGTTT
GGCAACCCTGTCATACCTTTTAAGGATGGTATTTATTTTGCTGCCACAGAGAAATCAAATGTTGTCCGTGGTTG
GGTTTTTGGTTCTACCATGAACAACAAGTCACAGTCGGTGATTATTATTAACAATTCTACTAATGTTGTTATACG
AGCATGTAACTTTGAATTGTGTGACAACCCTTTCTTTGCTGTTTCTAAACCCATGGGTACACAGACACATACTAT
GATATTCGATAATGCATTTAATTGCACTTTCGAGTACATATCTGATGCCTTTTCGCTTGATGTTTCAGAAAAGTC
AGGTAATTTTAAACACTTACGAGAGTTTGTGTTTAAAAATAAAGATGGGTTTCTCTATGTTTATAAGGGCTATC
AACCTATAGATGTAGTTCGTGATCTACCTTCTGGTTTTAACACTTTGAAACCTATTTTTAAGTTGCCTCTTGGTAT
TAACATTACAAATTTTAGAGCCATTCTTACAGCCTTTTCACCTGCTCAAGACATTTGGGGCACGTCAGCTGCAGC
CTATTTTGTTGGCTATTTAAAGCCAACTACATTTATGCTCAAGTATGATGAAAATGGTACAATCACAGATGCTGT
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>bDPP4 (yellow marked the nucleotides pre-destined by the primer used for isolation)

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>bDPP4-GFP

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G

10 Affidavit

I herewith declare that I autonomously carried out the thesis with the title “Interspecies-Transmission of animal Coronaviruses”.
No third party assistance has been used. I did not receive any assistance in return for payment by consulting agencies or any person. No one received ana kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.
I conducted the project at the following institution:
Institute of Virology, University of Veterinary Medicine Hannover.
The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.
I hereby confirm the above statements to be complete and true to the best of my knowledge.

Hannover, 05.09.2013,Tim Gützkow: _____

11 Acknowledgments

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Working at the Institute was fun and pain as most things in life are, but in the end I will look back at all the great time we had. It is in the nature of this academic training that there is continuously change in staff and most of the people I spent time with already went on to the next endeavour. Nonetheless, I am very grateful to have been a part of this Institute and want to thank everybody for being such a friendly and supportive community!

Beside all the mentioned people I especially want to thank Tina. Without her constant support and sympathy for the struggle of Ph.D. students it would have been a lot harder. She makes the better half of me.

Ich möchte an dieser Stelle aber auch vor allem meine Mutter danken, die zwar kein Englisch lesen kann aber nicht unerwähnt bleiben darf. Ohne ihren festen Glauben an mich, sowie die vielen Opfer die sie bereit war auf sich zu nehmen um mir diese Ausbildung zu ermöglichen, wäre es wohl nicht möglich gewesen.